



# The effects of immunoageing on the susceptibility to cigarette smoke induced COPD

Dissertation  
zum Erwerb des Doktorgrades der Medizin  
an der Medizinischen Fakultät  
der Ludwig-Maximilians-Universität zu München

vorgelegt von:  
Stefanie Günter

aus  
Rostock

2017



**HelmholtzZentrum münchen**

Deutsches Forschungszentrum für Gesundheit und Umwelt



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Mit Genehmigung der Medizinischen Fakultät  
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## Abstract

The pathogenesis of chronic obstructive pulmonary disease (COPD) is known to be closely linked to cigarette smoke exposure, or to be more precise, to the abnormal inflammatory response caused by inhalation of cigarette smoke or, rarely, of other inhalative noxes. The effects of cigarette smoke most often include the development of emphysema and chronic bronchitis leading to decline in lung function and subsequently to the loss of life quality and life time. Chronic inflammation, which plays a major role in the development of COPD can mainly be attributed to progressive activation of immune cells, which increases with exposure time. It was also shown that immune function changes with age, sometimes leading to overreactions. These changes on the one hand lead to the increase of autoimmune diseases, on the other hand they also predispose to immunodeficiencies. But even now, the immune system and especially its changes with age are still not fully understood.

Since increased inflammatory response plays an important role in the development of COPD, we hypothesized that in a mouse model for the development of COPD by chronic cigarette smoke exposure, the pathogenesis of COPD is characterized by an elevated immune response in aged mice compared to young animals.

To proof our hypothesis, female C57BL/6 mice aged 2 and 12 months were exposed to cigarette smoke with a concentration of 500 mg/m<sup>3</sup>, using a smoking chamber to create full body exposure and mimic human smoking habits. Exposure was performed twice a day for 50 min for three months. Control animals were handled, but only exposed to filtered air. Differential cell counts and inflammatory cell recruitment were performed using FACS on bronchoalveolar lavage and homogenates of whole lung tissue respectively. Other parameters for disease progression that were considered were lung function and emphysema development (by use of a stereological tool). We have found a decline in lung function due to cigarette smoke in old animals after 3 months of cigarette smoke exposure (demonstrated by an increase in lung compliance), while there were still no changes in lung function of the younger animals. Further analysis also showed an enlargement of alveolar space and an increase in airway collagen in old cigarette smoke exposed mice, that could not be found in the younger animals. Furthermore, we discovered an increase in tertiary lymphoid structures (iBALT) in the lung tissue of old cigarette smoke exposed animals only, which led to our hypothesis, that the changes in lung structure and function occurred due to immunoageing. Therefore, we analysed different cell lines to find changes due to cigarette smoke exposure and age. Differential cell counts of BAL cytopins revealed significantly higher lymphocyte levels in aged animals, whereas comparable increases in macrophage and neutrophil numbers for all CS-exposed mice were observed. In old animals, we found an increase in Th17 positive T-helper cells due to cigarette smoke exposure. An increase in the amount of these cells has been described to show a strong correlation with decline of lung function. Additionally, staining for MMP12 in lung tissue revealed significantly higher macrophage accumulation and activation in CS-exposed aged mice, which was in accordance with increased MMP12 mRNA expression and an elevated MMP12/TIMP1 ratio.

COPD-like changes in the lungs of mice were only observed in the lungs of the older animals at this timepoint. Correspondingly macrophage levels as well as Th17 cell levels were only increased in the older cigarette smoke exposed animals. This leads us to the conclusion, that

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the difference in lung damage due to cigarette smoke exposure in old vs. young animals can indeed be explained by differences in the immune reaction.

## Zusammenfassung

Es ist bekannt, dass das Auftreten der chronisch obstruktiven Lungenerkrankung (COPD) eng mit dem Zigarettenrauchen verknüpft ist. Um genauer zu sein, ist es zurückzuführen auf die abnormale Immunantwort auf den Zigarettenrauch, oder selten auch auf andere inhalatorische Noxen. Die Folgen von Zigarettenrauchen sind unter anderem die Entwicklung von Lungenemphysem und chronischer Bronchitis, die zu einer Verschlechterung der Lungenfunktion und damit zu einem Verlust von Lebensqualität und Lebenszeit führen. Die chronische Entzündung, die eine der Hauptrollen in der Entwicklung der COPD spielt, ist vor allem der progressiven Aktivierung von Immunzellen zuzuschreiben. Diese steigt mit der Expositionszeit. Es wurde bereits gezeigt, dass das Immunsystem sich im Alter verändert, und teilweise überreagiert. Ein Beispiel hierfür ist das häufigere Auftreten von Autoimmunerkrankungen im Alter. Andererseits prädisponiert ein höheres Alter auch für Immundefekte. Allerdings sind das Immunsystem und vor allem seine Veränderungen mit dem Alter noch immer nicht gänzlich erforscht.

Da eine gesteigerte Immunantwort eine wichtige Rolle in der Entwicklung der COPD spielt, haben wir angenommen, dass in einem Mausmodell die Exposition mit Zigarettenrauch zu der Entwicklung einer COPD führt. Die Pathogenese der COPD sollte durch eine gesteigerte Immunantwort der älteren Tiere im Vergleich mit den Jüngeren charakterisiert sein.

Um unsere Hypothese zu prüfen haben wir weibliche C57BL/6 Mäuse im Alter von 2 und 12 Monaten Zigarettenrauch mit einer Konzentration von  $500 \text{ mg/m}^3$  ausgesetzt. Hierfür haben wir eine Rauchkammer benutzt, um eine Exposition des ganzen Körpers der Mäuse zu erreichen und damit menschliches Rauchverhalten möglichst genau abzubilden. Die Exposition wurde 3 Monate lang, zweimal täglich für je 50 min durchgeführt. Die Kontrolltiere wurden umgesetzt, aber nur gefilterter Luft ausgesetzt. Differenzierte Zellzählungen und die Analyse von Entzündungszellen wurde mittels eines FACS-Geräts an der bronchoalveolaren Lavage und dem homogenisierten Lungengewebe durchgeführt. Die anderen Parameter, die für die Analyse des Fortschreitens der Erkrankung herangezogen wurden, waren die Bestimmung von Lungenfunktion und die Emphysementwicklung (diese wurde mit Hilfe eines stereologischen Werkzeugs bestimmt).

Nach 3-monatiger Exposition fanden wir eine Verschlechterung der Lungenfunktion durch Zigarettenrauch bei den älteren Tieren nach 3 Monaten Exposition (was sich durch einen Anstieg der Lungencompliance zeigte), während sich bei den jungen Tieren noch keine Veränderungen zeigten. Weitere Untersuchungen zeigten eine Vergrößerung der Alveolarräume, sowie die Vermehrung von Collagen in den Wänden der Atemwege. Diese Veränderungen ließen sich bei den jüngeren Tieren nicht nachweisen. Zudem fanden wir eine Vermehrung von tertiären lymphoiden Strukturen (iBALT), die sich nur im Lungengewebe der älteren berauchten Mäuse nachweisen lässt. Dies führte uns zu der Hypothese, dass die Veränderungen von Lungenstruktur und -funktion durch die altersbedingten Veränderungen des Immunsystems bedingt sind. Daher überprüften wir verschiedene Zelllinien auf durch Alter und Zigarettenrauch bedingte Unterschiede. Differenzierte Zellzählungen aus der BAL zeigten signifikant erhöhte Lymphozyten-Level bei den älteren Tieren, während die Vermehrung von Makrophagen und Neutrophilen in allen berauchten Gruppen zu finden war. Bei den älteren Tieren fanden wir einen durch Zigarettenrauch verursachten Anstieg der Th17 positiven T-Helfer Zellen. Eine Vermehrung dieser

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Zellen zeigte eine starke Korrelation mit einer Verschlechterung der Lungenfunktion. Die immunohistochemische Färbung des Lungengewebes mit einem MMP12-Antikörper zeigte eine erhöhte Rekrutierung und Aktivierung von Makrophagen bei berauchten Mäusen. Dies zeigte sich auch in einer erhöhten MMP12-mRNA Expression und einem erhöhten MMP12/TIMP1 Verhältnis.

COPD-typische Veränderungen zeigten sich zum Untersuchungszeitpunkt nur in den Lungen der berauchten älteren Mäuse. Damit übereinstimmend fanden wir nur bei den älteren berauchten Mäusen eine Vermehrung von Makrophagen und Th17-Zellen. Dies führt uns zu dem Schluss, dass die unterschiedlichen Ausmaße der Lungenschädigung durch Exposition mit Zigarettenrauch bei älteren und jüngeren Mäusen durchaus durch Unterschiede in der Immunreaktion erklärt werden können.

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# 1 Introduction

## 1.1 Chronic obstructive pulmonary disease (COPD)

### 1.1.1 Definition and diagnosis of COPD

COPD is a disease that is defined by three main pathological characteristics: the limitation of airflow, the development of emphysema and the chronic inflammation of airways [1].

For the clinical diagnosis of COPD, the most important parameter is airflow limitation. For clinical diagnosis a forced expiratory volume (the maximum volume the patient can exhale in one second =  $FEV_1$ ) of less than 70% of the vital capacity of the patient is sufficient to diagnose COPD. The severity of the disease is further characterized by  $FEV_1$  of the patient compared to a healthy reference group adjusted to gender, height and weight according to the classification of the GOLD stages (see table 1) [2].

The treatment of COPD requires inter alia the reduction of exacerbations. Exacerbations

GOLD stage		Severity of air flow limitation
GOLD 1	mild	$FEV_1 \geq 80\%$ of reference value
GOLD 2	moderate	$50\% \leq FEV_1 < 80\%$ of reference value
GOLD 3	severe	$30\% \leq FEV_1 < 50\%$ of reference value
GOLD 4	very severe	$FEV_1 < 30\%$ of reference value

**Table 1:** Characterisation of disease stage according to air flow limitation as described in [2].

are defined as “acute events characterized by a worsening of a patient’s respiratory symptoms that is beyond normal day-to-day variation and leads to a change in medication”[2]. The likelihood of exacerbations is a factor to be regarded in the assignment to therapeutic groups. The patient’s risk group is determined by the amount of exacerbations and their lung function classification. Patients characterized as low risk have a lung function that classifies them as GOLD stages 1 or 2 and  $\leq 1$  exacerbations per year. These exacerbations must not require hospitalisation. Patients characterized as high risk have a lung function classifying as GOLD stages 3 or 4 and have  $\geq 2$  exacerbations per year. In addition, one exacerbation that requires hospitalisation necessarily classifies the patients into the high risk group.

Another parameter that defines the line of therapy is the amount of symptoms. For the classification of symptoms there are two scales: The mMRC-scale (see table 2) and the COPD-Assessment-Test-scale (the CAT questionnaire can be taken from [3]). Patients reaching 0-1 points on the mMRC scale or  $< 10$  points on the CAT scale are classified as less symptomatic patients. Patients reaching  $\geq 2$  points on mMRC or  $\geq 10$  points on the CAT scale are classified as more symptomatic.

According to these parameters, patients are distributed into different treatment groups as seen in figure 1.

Grade	Symptoms
Grade 1	Patient's breath is as good as the healthy reference group
Grade 2	Patient has difficulties climbing hills or stairs
Grade 3	Patient is able to walk at his own speed for about a mile or more
Grade 4	Patient is able to walk more than about 100 yards on the level.
Grade 5	Patient is unable to leave his house or breathless while talking

**Table 2:** mMRC-scale according to [4]

<b><u>Group A</u></b> Low risk Less symptoms	<b><u>Group B</u></b> Low risk More symptoms
<b><u>Group C</u></b> High risk Less symptoms	<b><u>Group D</u></b> High risk More symptoms

**Figure 1:** Combined Assessment of COPD according to GOLD [2]

### 1.1.2 Epidemiology of COPD

According to WHO reports [5], COPD has been found to be the fourth leading cause of death worldwide. It is estimated to be the third leading cause of death by 2030. This makes the analysis of risk factors and pathomechanisms a very important task for the future of medical treatments. Even though other risk factors for the development of COPD have been described, cigarette smoking is still the most important one in developed countries. Lundbäck et al. [6] have conducted a study showing a significant increase in COPD cases for cigarette smokers compared to non-smokers. They have also revealed an age dependent increase in the prevalence of COPD in both, the smoking group and the non smoking group. The percentages of patients with COPD in the study, depending on age and smoking habits are given in table 3. The amount of ex-smokers diagnosed with COPD has been shown to range between that of smokers and non-smokers.

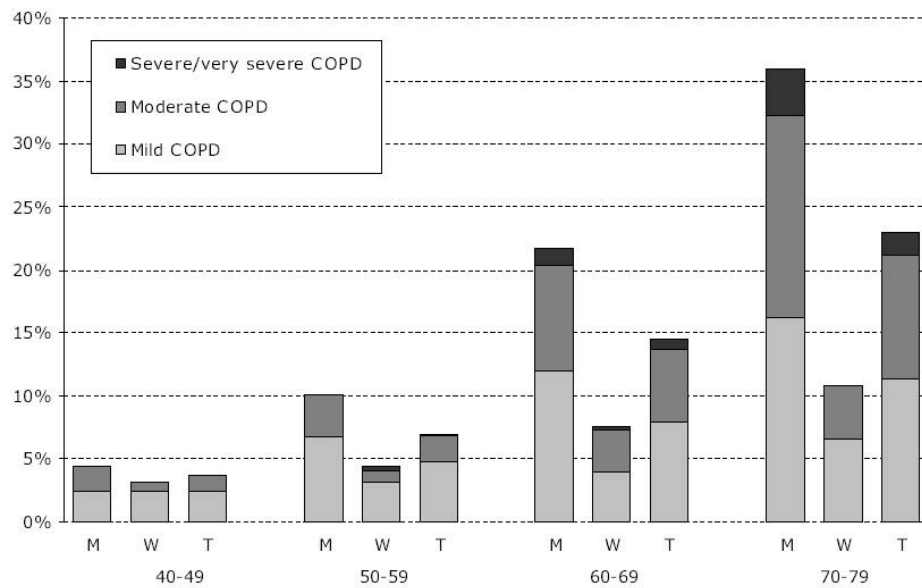
Ageing additionally seems to be a predisposing factor for the development of COPD. This

Age	Non-Smokers	Smokers
46-47	1%	5%
61-62	2%	24%
76-77	16%	45%

**Table 3:** Table of COPD prevalence in % depending on age group and smoking habits, generated with the data presented in [6]

has been shown in epidemiological analyses by an age dependent increase in the percentage of





**Figure 2:** COPD prevalence according to age group and gender [7], M= male , W=female, T= total

patients diagnosed with COPD. An increase with ageing has also been found in the severity of the disease, as defined by the GOLD stages (see figure 2) [7]. Even though lung function physiologically declines during the process of ageing, there are significant differences between smokers and non smokers in the development of COPD.

COPD is a very serious lung disease, as the patients suffer from a significant impairment to their everyday lives. It has been shown that patients suffering from COPD have a significantly decreased 6 minute walking distance compared to subjects not suffering that disease. It has also been determined that COPD patients have a lower Barthel-index, which is an indicator of how much a person is able to cope in their everyday life and care for themselves [8].

However, patients with COPD do not only suffer from a significant loss of life quality, but also of lifetime. Feenstra et al. have discovered that male COPD patients lose an average of 8 years off their lifetime, while female COPD patients lose a total of 10.5 years off their life expectancy compared to people without a COPD diagnosis [9].

### 1.1.3 Pathology of COPD

#### 1.1.3.1 Small airway remodelling

Small airway wall thickness has been reported to have a strong correlation with  $FEV_1$ , which is the most relevant clinical parameter for the diagnosis of COPD.  $FEV_1$  defines the stages 1-4 in the GOLD classification. It has been shown, that increasing severity of clinical symptoms and advancing stages, correlate with an increase in the thickness of small airways [1]. A correlation has also been found between small airway wall area percentage and clinical severity of the disease. Another parameter that closely correlates with disease severity is the “percentage of occlusion of the fully expanded airway lumen by inflammatory exudates containing mucus”[10]. Using this correlation, one could also classify the inner luminal area of small airways as a parameter closely correlated to  $FEV_1$ . These correlations have been proven both by histological analysis of lung tissue from patients and in measurements taken from computertomographic

(CT) images [10],[11].

In addition to the increase of airway thickness with the progression of the disease, it has also been demonstrated that in COPD patients the amount of micro vessels in the walls of small airways is significantly increased compared to the healthy control group [12].

### 1.1.3.2 Emphysema development

Emphysema is one of the foremost terms that comes to mind when talking about COPD. However, only a weak correlation between lung function analysis, in particular FEV<sub>1</sub>, and emphysema exists. This is not surprising, as FEV<sub>1</sub> is determined by the airway area. None the less, emphysema development severely reduces the diffusion capacity of the lung, as it reduces the alveolar surface area. Emphysema is classically determined by pathological methods. Radiologic methods such as CT have taken over the diagnostic process in most cases. There has been proven to be a good correlation between CT analysis values for emphysema and pathological analysis [13].

It has been found that emphysematous lesions can be induced by intratracheal instillation of human elastase into animal lungs [14]. These findings lead to the assumption, that emphysema in humans can be caused by an imbalance of elastases and anti-elastases in the lung. This theory is supported by the fact, that humans with  $\alpha_1$ -antitrypsin deficiency develop emphysema independent of cigarette smoke exposure. But even without this genetic defect smokers develop emphysema. In the analysis of bronchoalveolar lavage fluid from smokers, a chronic increase in elastase levels as well as in MMP-levels (matrix-metalle-proteinases) could be found. Additionally an acute increase in neutrophil elastase activity following cigarette smoke exposure has been found [15]. In addition to the increase in elastase levels a decrease in the levels of anti-protease activation could be found in patients with COPD [16]. But even in healthy smokers the activity of  $\alpha_1$  antitrypsin is reduced in comparison to non smokers. This is suggested to be caused by the inactivation of  $\alpha_1$ -antitrypsin by methionine sulfoxide [17]. An analysis of emphysema severity (analysed by CT imaging) and the comparison to elastase and antielastase levels in BAL have shown a direct correlation between elastase levels and emphysema severity. Anti-elastase levels on the other hand have shown an indirect correlation to the severity of the disease [18].

Interestingly women have been found to be more susceptible to cigarette smoke induced COPD, especially in the early stages of COPD. The latter has been demonstrated by a greater lung function reduction as well as a more severe disease compared to men after exposure to the same amount of cigarette smoke [19]. In contrast to those findings a greater emphysema development in men compared to women at the same stage of COPD has been discovered [20].

### 1.1.3.3 Chronic bronchitis and tertiary lymphoid structures

COPD has been known to cause not only airflow limitation, but also chronic inflammation of the airways. Chronic bronchitis usually manifests in chronic coughing as well as in increased sputum production. In patients with chronic bronchitis pulmonary biopsies have shown an increased number of total leukocytes in comparison to healthy control subjects. This increase is mostly due to an increase in macrophages and T-lymphocytes [21]. In patients that additionally suffer from airflow limitation, an increase in macrophages could be detected that was significant even compared to patients suffering from chronic bronchitis only. It has been found that the amount of inflammatory cells such as CD8 positive T-cells, neutrophilic and eosinophilic cells show a positive correlation with the clinical severity of COPD [22].

The inflammatory cells in airways have been found to mostly be organized in lymphoid follicles

[1]. These so called iBALT (inducible Bronchus Associated Lymphoid tissue) structures have been described to consist of B-cell follicles as well as T-cell areas. iBALT structures seem to support B- and T-cell proliferation [23]. Inflammation of airways has been described to persist in patients with COPD even after one year of smoking cessation. In smokers that were asymptomatic before smoking cessation however, inflammation of the airways decreases [24].

### 1.1.3.4 Pulmonary hypertension

Pulmonary arterial hypertension is a common complication in patients with COPD especially in patients of stages III and IV according to GOLD. It has been unveiled that 36% of these patients develop pulmonary hypertension. Pulmonary hypertension in COPD patients is also associated with a lower survival rate [25].

In patients with pulmonary hypertension alterations in arterial structure have been found in comparison to control groups. Analysis of medium and larger muscular arteries have shown increased percentages of media and intima compared to the healthy control groups. Additionally a trend towards an increase in muscle in the media of smaller arteries has been detected [26]. Furthermore, IL 6 seems to play a role in the development of pulmonary hypertension as a correlation between IL 6 levels and the degree of pulmonary hypertension has been found [27]. Even though there does not seem to be a correlation between pulmonary hypertension and either lung function or emphysema development, a correlation between the increase in pulmonary blood pressure and a decrease in PaO<sub>2</sub> has been found. Patients with pulmonary hypertension also seem to suffer from more severe dyspnoea compared to patients with the same or even more airway obstruction with normal pulmonary blood pressure [27], [28].

It has been suggested that long term oxygen therapy in patients with severe COPD and pulmonary hypertension may decrease pulmonary arterial blood pressure, even though the values do not return to baseline levels [29].

### 1.1.4 Therapeutic approaches to COPD

The treatment of COPD patients is given according to the needs of the patient. The patients are distributed into groups ranging from A to D defined by GOLD [2] as described in chapter 1.1.1. An overview over the treatment options for COPD was described in [30]. The guidelines for treatment of COPD given by GOLD is presented in figure 3.

#### 1.1.4.1 Smoking cessation

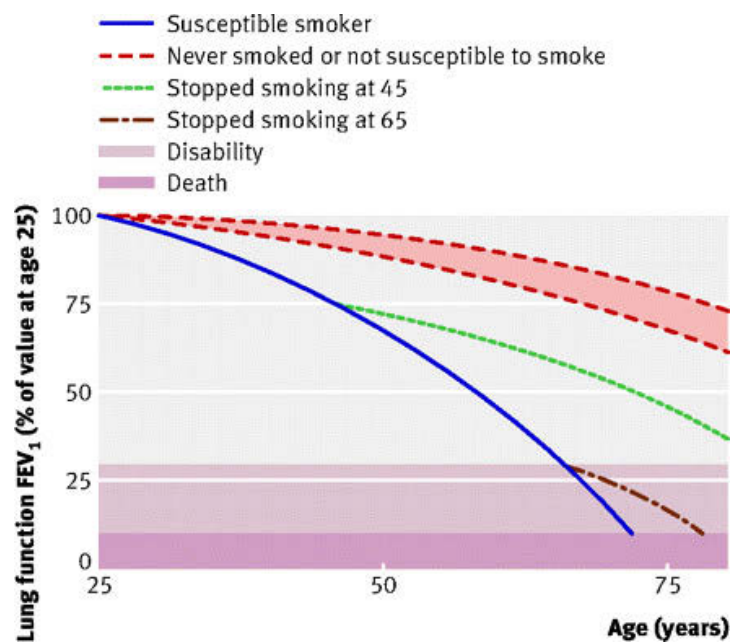
It has been known for a long time, that smoking is the main risk factor for the development of chronic obstructive pulmonary disease. It accounts for approximately 80% of all COPD related deaths [31]. Therefore, prevention of disease development and exacerbations by terminating exposure to the main risk factor is an essential element in the therapeutic approach. It has been shown that after smoking cessation, lung function in COPD patients declines at a much slower rate, similar to the physiological age dependent decline, than in the patients that continue smoking [32], [33]. The rates of lung function decline in smokers and non-smokers are demonstrated in figure 4.

Smoking cessation has also been shown to reduce airway reactivity which leads to an increase in FEV<sub>1</sub>, which has been found to be the primary marker for lung function in COPD [35].

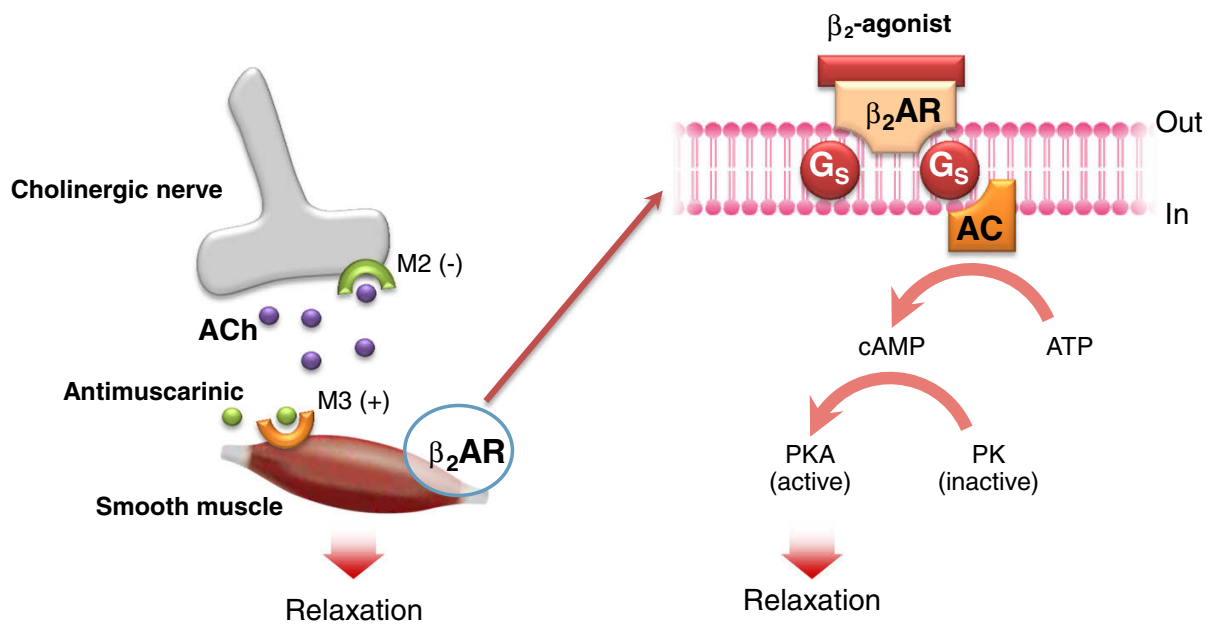
A	B	C	D
Short acting $\beta_2$ agonists and/or short acting anticholinergics			
Long acting $\beta_2$ agonists and/or long acting anticholinergics			
Theophylline			
	Long acting $\beta_2$ agonists or long acting anticholinergics		
		Long acting $\beta_2$ agonists and long acting anticholinergics	
		PDE4-Inhibitors and Long acting $\beta_2$ agonists or long acting anticholinergics	
			Inhaled Glucocorticosteroids and Long acting $\beta_2$ agonists and/or long acting anticholinergics
			ICS and Long acting $\beta_2$ agonists or long acting anticholinergics and PDE4-Inhibitors
			Carbocysteine N-Acetylcysteine

■ First line Therapy  
■ Second line Therapy  
■ Alternative Therapy

**Figure 3:** Treatment guidelines according to GOLD [2]



**Figure 4:** Lung function decline due to ageing and cigarette smoke. According to figure 1 in [33], taken from [34]



**Figure 5:** Mechanisms of bronchodilation (picture taken from [39])

#### 1.1.4.2 $\beta_2$ -agonists

One of the approaches to symptomatic treatment of COPD is bronchodilation. There are two main kinds of bronchodilators that are used in the standard therapy according to the GOLD guidelines for COPD treatment. The most commonly used group is  $\beta_2$  agonists that can be further divided into the groups of short acting and long acting  $\beta_2$  agonists (see table 4). Short acting  $\beta_2$  agonists have been shown to significantly increase both FEV<sub>1</sub> as well as FVC (forced vital capacity) in patients with COPD [36]. Studies have shown that under therapy with

short acting $\beta_2$ agonists	long acting $\beta_2$ agonists
Salbutamol	Formoterol
Fenoterol	Salmeterol
Terbutalin	Indacaterol (ultra long acting)
Reproterol	
Albuterol	

**Table 4:** List of  $\beta_2$  -agonists

long acting  $\beta_2$  agonists symptoms in COPD patients decrease by a greater amount than with the use of short acting  $\beta_2$  agonists [37] when prescribed in combination with anticholinergics. No differences have been found for the onset times between short acting and long acting  $\beta_2$  agonists. Thus, long acting  $\beta_2$  agonists are preferred as the baseline therapy [38].

#### 1.1.4.3 Anticholinergics

Anticholinergics are the second group of bronchodilators used in the therapy of COPD. While  $\beta_2$  agonists play a role in the active relaxation of airway smooth muscle cells, anticholinergic drugs prevent smooth muscle cells from contracting due to parasympathetic stimulation (see figure 5).

Anticholinergics such as Ipratropium or Tiotropium have been shown to have an additional effect in when used in combination with  $\beta_2$  agonists. This effect can be seen in an increase of both the FEV<sub>1</sub> and the FVC [40]. These effects seem to be even stronger with Tiotropium compared to Ipratropium [41]. In addition to the short term effect of bronchodilation, Ipratropium has been shown to increase lung function baseline levels for both values (FEV<sub>1</sub>, FVC). This effect is greater in patients that quit smoking and can not be seen in this amount in current smokers [42]. Another effect of Ipratropium treatment is an increased oxygen saturation during the night as well as a better sleep quality combined with prolonged REM phases [43].

### 1.1.4.4 Inhaled glucocorticosteroids

In more severe cases of COPD inhaled glucocorticosteroids (ICS) are an important part in the therapeutic approach, even more so in patients that suffer from frequent exacerbations.

ICS have been shown to increase FEV<sub>1</sub> during the first few months of treatment. This effect is strongest in ex-smokers and women. After this time however, lung function starts to decline in the same manner as in the placebo controlled group [44]. There is conflicting data on whether inhaled glucocorticosteroids have a significant effect on mortality. A recent overview [45] has shown no difference in mortality in several double blind studies, while in other studies such as [46], a reduced mortality rate has been found. In addition, inhaled glucocorticosteroids have been described to reduce exacerbations [45], [47]. There is however a higher rate of pneumonia in patients treated with ICS alone or in combination with  $\beta_2$  agonists than in patients treated with  $\beta_2$  agonists alone [48].

This is one of the main reasons why they are only used to treat patients with moderate and severe COPD.

### 1.1.4.5 Phosphodiesterase-Inhibitors (PDE4-Inhibitors)

A relatively new development in the treatment of COPD is the use of PDE4-Inhibitors such as Roflumilast and Cilomilast. In placebo controlled trials these PDE4-Inhibitors have been shown to increase FEV<sub>1</sub> as well as FVC. Furthermore reduced exacerbation rates have been found [49], [50]. An increase in lung function values has also been demonstrated in the combined treatment with both  $\beta_2$  agonists as well as in combination with anticholinergics. The increase in lung function with the combined treatment is significant compared to the treatment with either substances alone [51].

### 1.1.4.6 Other possible treatment options

#### Methylxantines

Theophylline is a drug only used as a third line therapy in all stages of COPD. It has been shown to improve lung function in COPD patients, but to be less effective here than long acting  $\beta_2$  agonists. It nevertheless has been found to show significant effects when used in addition to  $\beta_2$  agonists. [52]. Low dose Theophyllin in combination with ICS has shown a significantly greater increase in FEV<sub>1</sub> than either substance alone [53].

#### Mucolytic Substances

The mucolytic substances used in the treatment of COPD according to GOLD [2] are N-Acetylcysteine and Carbocysteine. These substances have not shown any improvement in lung

function such as FEV<sub>1</sub> or FVC, but in patients not treated with ICS treatment with mucolytic substance has shown to decrease the rate of exacerbations [54], [55].

### **Physiotherapy**

Patients with COPD benefit from dedicated physiotherapy. Physiotherapy has been shown to increase FVC as well as 6 minute walking distance. It also decreases subjective dyspnoe and reduces the rate of exacerbations [56].

## **1.2 Inflammatory response**

### **1.2.1 The role of Inflammation in COPD Development**

#### **1.2.1.1 Innate immune system**

The innate immune system is usually the first one to react to stimuli. The cells from the innate system that have the greatest effect on COPD development are neutrophils and macrophages.

### **Neutrophils**

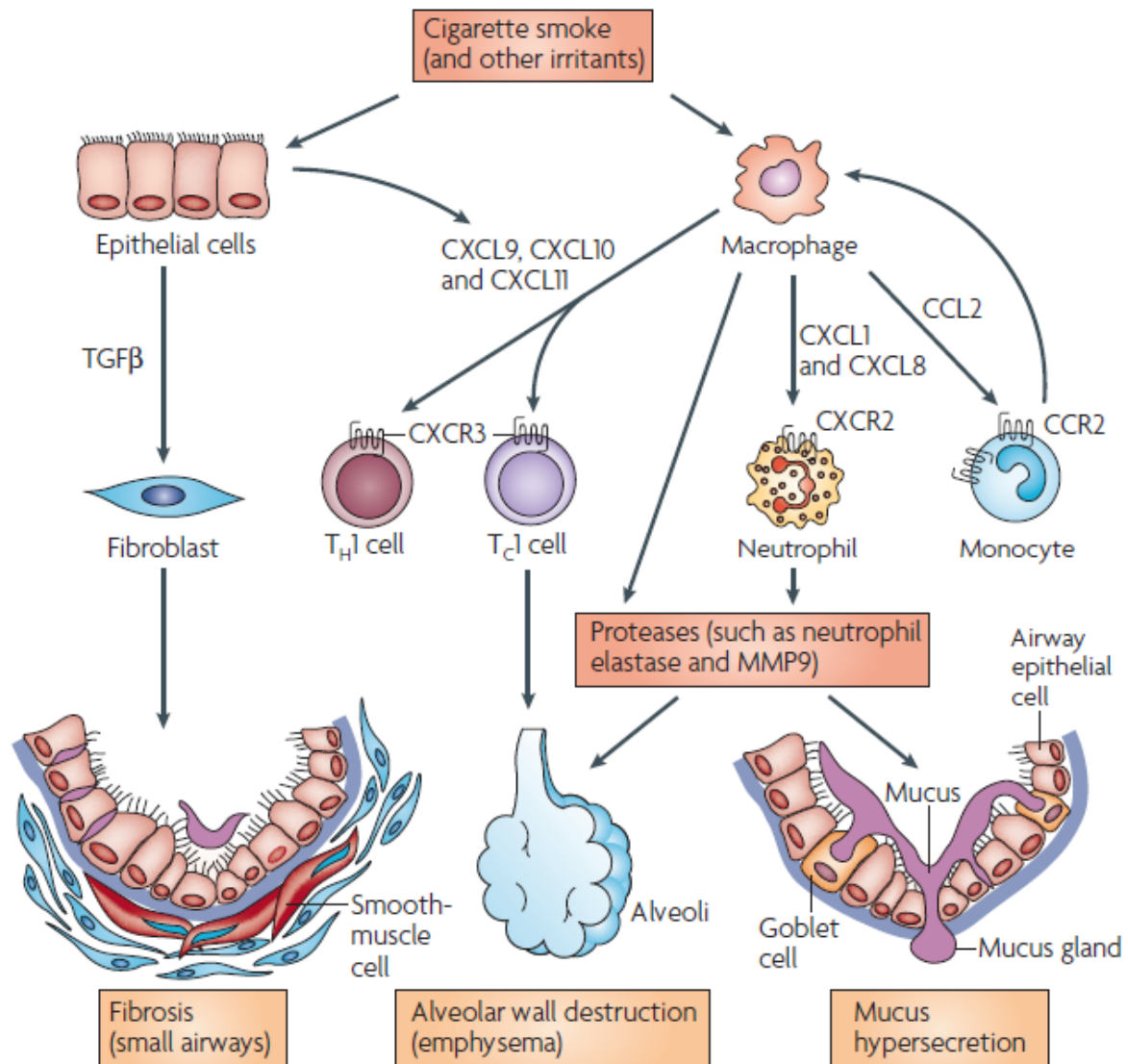
Neutrophilic granula contain several degrading proteins. The most commonly known one to play a role in the development of COPD is neutrophilic elastase. In addition also proteinase-3 (PR-3) is produced in neutrophils and has been found to have an even greater effect in emphysema development in hamsters than elastase [58]. However, only a weak correlation between emphysema development and lung function has been described [13]. It is thus not surprising that even though the number of neutrophils in sputum is increased in patients with COPD [59] there is only a weak inverse correlation between the neutrophil count in bronchoalveolar lavage (BAL) and either FEV<sub>1</sub> or the health status of patients. No association between neutrophil counts and emphysema, exacerbation rates or systemic inflammation has been found [60]. Gene expression of chemoattractants for neutrophilic migration were up regulated shown by several markers such as epithelial-derived neutrophil attractant-78 (CXCL5), interleukin-8, LTB<sub>4</sub>, CXCR1 and CXCR2 [61]. In addition, some of these markers were found to increase in case of COPD exacerbations such as LTB<sub>4</sub> and 8-isoprostane. No correlation between airway obstruction (determined by FEV<sub>1</sub>) and the concentration of the markers mentioned above, could be determined [62].

8-isoprostane has been described to increase IL-8 expression in human macrophages [63].

In conclusion, it has been shown that neutrophils play an important role in the development of emphysema. Since emphysema however has little effect on FEV<sub>1</sub>, these effects often are underrated.

### **Macrophages**

Macrophages however seem to play the more important role in the development of COPD. Severe emphysematous destruction of lung tissue has been shown to be associated with an increase of the absolute numbers of macrophages [64]. It has been demonstrated that MMP12 (matrix-metalloproteinase 12) deficient mice fail to develop emphysema after cigarette smoke exposure



**Figure 6:** Immune cells in the development of COPD taken from the review by Barnes [57], where an overview for the roles of different cell types was given. Figure reprocessed from [57].



[65]. DiStefano et al. have shown an increased number of macrophages in bronchoalveolar lavage (BAL) of COPD patients. In addition an inverse correlation between the number of macrophages and FEV<sub>1</sub> was discovered [59]. MMP12 seems to be one of the most important factors in emphysema development. Thus, not only the number of total macrophages in the lung is important, but also their activation which was determined by MMP12 expression is. It has been shown that the number of MMP12 expressing macrophages was increased in both BAL samples as well as in biopsy samples, which was determined by immunohistochemical staining in COPD patients. Furthermore the intensity of the immunohistochemical staining was greater than in control samples [66].

Macrophages can differentiate into two subtypes, M1 and M2 macrophages. In COPD patients a suppression of M1 macrophages has been found. These changes seem to progress with COPD development [67]. Concurring with these findings, an increase in M2 macrophages has been found in COPD patients compared to healthy control group. A correlation between the amount of M2 macrophages and disease severity has been found [68].

### 1.2.1.2 Adaptive immune system

In addition to the innate immune system, the adaptive immune system is another important factor in the development of COPD. The main cell types of the adaptive system are B- and T-lymphocytes.

#### T-Cells

T-cells are mainly divided into CD8-positive T-suppressor cells and cytotoxic T-cells as well as CD4-positive T-helper cells. In (non smoking) COPD patients an increase in percentage of CD8+ lymphocytes was found in comparison to healthy patients. In addition, a correlation between a higher CD4:CD8 ratio and a higher FEV<sub>1</sub> could be established [69]. Further investigation of subtypes of CD8 positive cells in bronchoalveolar lavage fluid obtained from COPD patients revealed a higher number of naive T-cells as well as a lower number of memory T-cells in comparison to smokers that show no symptoms of COPD. The comparison to healthy smokers shows that these changes only occur in patients that are susceptible to cigarette smoke and are not a general reaction to smoking. The changes in naive and memory T-cells seem to only occur in lung tissue as peripheral blood samples exhibit no differences between healthy smokers and COPD patients in the numbers of either of those cells [70].

However, not only the differences in the numbers of various subtypes of T-cells contribute to the changes in T-cell immunity occurring in the development of COPD. It has also been found that changes in T-cell activation due to chronic exposure to cigarette smoke affect disease progression. Experiments were conducted where CD3 positive T-cells were taken from wild type mice, exposed to chronic cigarette smoke, and transferred into immunodeficient mice. The latter animals then developed changes that are pathognomonic of COPD such as accumulation of macrophages and neutrophils. In addition, matrix degradation leading to airspace enlargement occurring in emphysema have been described. These changes occurred independent of cigarette smoke exposure [71].

One of the most affected subtypes in COPD development are regulatory T-cells. This cell type has been shown to be up regulated in lung tissue of COPD patients. T-regulatory cells were mainly located in lymphoid follicles [72]. The increase in T-reg. cells was also found in bronchoalveolar lavage fluid in COPD patients as well as in healthy smokers. Furthermore, the

increase was related to the number of pack years [73]. It has also been shown that regulatory T-cells can prevent the development of B-cell infiltrates due to cigarette smoke exposure [74]. The other group of T-cells that are important in the development of COPD are the T-helper cells. This group can be divided into several subgroups. One of these is the TH1 cells. An increase in percentage of TH1 cells has been found in COPD patients compared to a healthy control group. Along with these findings a decrease in TH2 cells has been discovered [75]. The increased TH1 cells however show a decreased response to bacterial infections, which leads to an increased rate of pulmonary infections in COPD patients [76].

The group of T helper cells that is now suspected to play the most important role in the development of COPD are TH17 cells. An increase of TH17 cells has been found in COPD patients, both compared to healthy smokers as well as to non smokers. A correlation has been found between the number of TH17 cells and the severity of airflow limitation and disease severity [77], [78].

### **B-Cells**

In addition to T-cells, B-cells contribute to the development of COPD. It has been unveiled that the numbers of B-cells were increased in patients with COPD compared to the healthy control group. An increase in B-cells in patients with COPD stage 2 compared to patients with COPD stage 3 has also been shown. Furthermore, the values of percentage of FEV<sub>1</sub> from FEV<sub>1</sub>-predicted correlate with the number of B-cells. A more severe airway hyper-responsiveness has been reported for patients with higher numbers of B-cells [79].

In patients with COPD as well as in a mouse model simulating COPD, B-cell follicles was found. The B-cells forming these follicles exhibit an oligoclonal antigen-specific reaction. In mice, the development of follicles were proven to progress over time. Additionally, the amount of follicles correlates with airspace enlargement [80]. In order to prove that B-cells are vital for the development of lymphoid follicles as well as for emphysema development, B-cell deficient mice were exposed to cigarette smoke in a chronic exposure simulation. In wild type mice an increase in inducible bronchus associated lymphoid tissue (iBALT) as well as an increase in lung compliance and airspace enlargement was visible. These changes could not be found in B-cell deficient mice. In addition, in vitro experiments have proven B cell-derived IL-10 to drive macrophage activation and to also lead to MMP12 up regulation [81].

### **1.2.2 Ageing and Inflammation**

In the previous chapters it has been described that the immune system plays an important role in the development of COPD. In chapter 1.1.2 it was established that COPD is a disease that mainly affects the elderly. Therefore, changes in the immune system in the course of senescence might be a main factor in COPD pathology. Changes to the immune response in aged animals have indeed been found. For example Kovacs et al. have shown a decrease in immunity in aged mice compared to young animals following burn injury, resulting in a higher mortality in aged animals [82].

### 1.2.2.1 Age related changes in the innate immune system

An overview over the age related changes in the innate immune system was given, e.g., in the review by A. Panda et al. [83].

#### Changes in neutrophils

Since neutrophils and their products play a key role in the development of emphysema, the changes in neutrophils with ageing of the immune system are a very important aspect to be considered. In bronchoalveolar lavage fluid of old and young healthy humans, it has been demonstrated that the number of neutrophils was increased in the older humans compared to the younger generation. In addition, the levels of both interleukin 8 as well as neutrophil elastase were elevated. On the other hand increases in  $\alpha_1$ -antitrypsin as well as elastase inhibitory capacity (EIC) have been described in the older test subjects [84]. As smoking was reported to decrease anti-elastase activity [17], the increased baseline elastase leads to greater susceptibility. Further investigation of changes in neutrophil function with ageing have not shown any changes in migration or adhesion under basal conditions as well as after stimulation. Changes in the activity of superoxide dismutase could not be found either. However, it has been unveiled that in older humans the chemotaxis of neutrophils has been severely attenuated [85]. Additionally, it has been shown that after stimulation with fMLP, superoxide production in elderly subjects has been reduced in comparison to young controls. The latter has been demonstrated for both, circulating as well as exudated neutrophils [85], [86]. Even though in some papers no changes in phagocytotic function have been found, Wenisch et al. have shown a reduction in the number of phagocytized *Escherichia coli* as well as of *Staphylococcus aureus* by aged neutrophils. Resting neutrophils also showed an increased intracellular calcium level with age as well as a reduced hexose uptake. In addition, a reduction in intracellular reactive oxygen production after stimulation with *S. aureus* has been found, leading to impaired bactericidal activity [87].

#### Changes in macrophages

The other important cell type in the innate immune system for the development of COPD is the macrophages. As with most cells, macrophages undergo deterioration in the process of ageing. Decreased function has been shown for chemotactic as well as phagocytotic activity [88]. In addition, superoxide anion production is compromised in the macrophages of old mice compared to young animals. Furthermore, the defenses to antioxidant stimuli are decreased, which can be seen by the decrease in superoxide dismutase activity with ageing [88]. Functional changes have neither been found for adherence capacity nor for the release of oxidant and inflammatory mediators. Examples of these unchanged mediators are extracellular superoxide anion, TNF- $\alpha$  and PGE<sub>2</sub>, as well as the oxidized glutathione/reduced glutathione ratio [88]. In addition to the changes in the cells themselves, also the external milieu impacts the function of macrophages. By incubating macrophages, taken from young rats, with serum from old animals, an impairment of the cells could be demonstrated. Young macrophages incubated with serum from old animals showed impaired capacity to induce tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) production. Furthermore, increased basal levels of interleukin-6 (IL-6) could be determined [89].

### 1.2.2.2 Age related changes in the adaptive immune system

A good overview over the age related changes in the adaptive immune system is, e.g., given in the review by Weng [90]

#### 1.2.2.2.1 Age related changes in T-cells

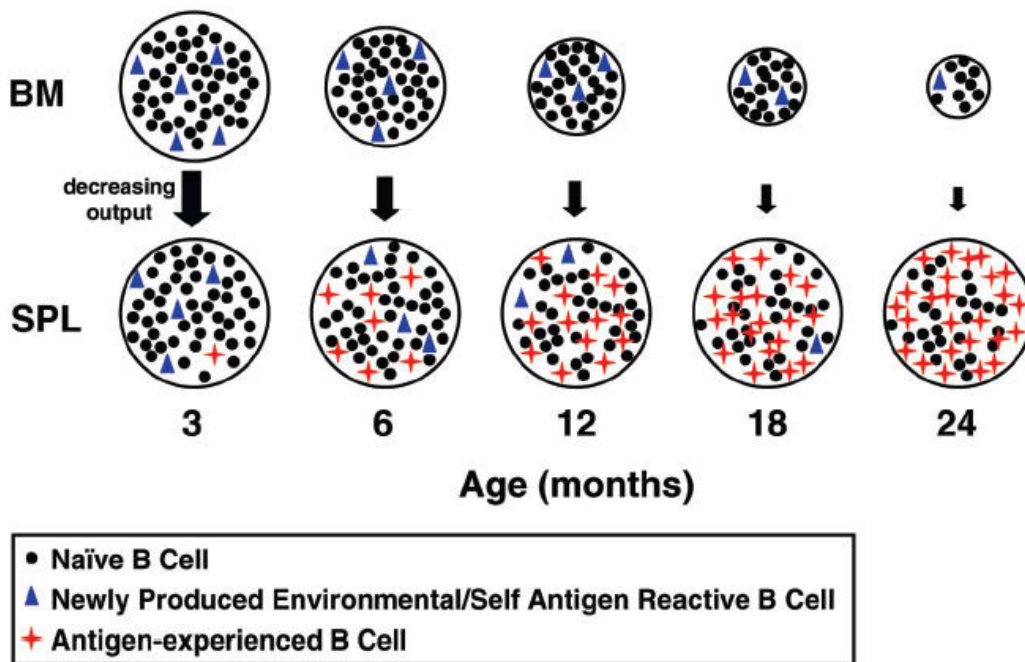
With most cell types of the immune system the first parameter to change is the number of cells. CD4+ T-helper cells from young healthy human subjects are predominantly composed of CD27 and CD28 positive naive cells. In aged subjects the number of these naive cells declines and is replaced by CD27 and CD28 negative memory T-helper cells [91]. A decrease in CD28 expressing T-helper cells in aged healthy test subjects has also been shown to correlate with a reduction of the CD4/CD8 ratio [92].

In addition to the changes in numbers, modifications in cell function with ageing have also been described [93]. These changes might be caused by the lower output of cells from the bone marrow, and therefore by a longer lifetime of the cells. This could indicate that the age of the cells themselves might be more important than the age of the individual. Haynes et al. have reported that cells taken from old mice show a decrease of expansion in culture as well as decreased expression levels of IL-2 compared to cells taken from young animals. To prove that the age of the cells is the main factor for such changes, both young and old animals were treated with an anti-CD4 antibody to destroy all T-helper cells. The cells sampled after this treatment showed a better response to stimuli in both groups, leading to the conclusion that a low output of T-lymphocytes is the main reason for the changes in function [93].

#### 1.2.2.2.2 Age related changes in B-cells

It has been reported that the number of circulating B-cells decreases with ageing [95]. While the absolute number of B-cells decreases, the changes in the sub-populations differ considerably. CD27 positive B-cells are memory cells that can transform into plasma cells. The total number of memory cells has been shown to be only marginally decreased in old individuals compared to young ones [96]. CD5 positive B-cells are defined as B1 cells that are involved in T cell-independent antibody production. The third important cell line in B-cells is the CD40+ cells that are responsible for T-dependent antibody responses. In absolute numbers, both CD5+ and CD40+ B cells are reduced in elderly subjects [96]. Regarding the percentages of the three sub-populations, a decrease in CD5+ B cells can be seen while the percentage in CD27+ B cells increases. The percentage of CD40+ B-cells does not change significantly with ageing [96]. In addition to the changes in B-cell numbers, also the production of antibodies changes with age. Increases in the levels of IgG and IgA have been described in aged subjects while there were no changes in IgM [95]. In addition, an analysis of IgG subclasses was performed, where an increase in IgG 1, 2 and 3 could be observed, while no changes were detected in IgG 4 levels [95]. These changes might be linked to a down-regulation of the E2A-encoded transcription factor E47 that has been observed in the B-cells of aged mice [97]. This transcription factor plays a role in class switch of antibodies as well as in somatic hyper-mutation. The down-regulation of E47 leads to reduced amounts of switched antibodies that are released by activated splenic B cells. Similar trends have been seen in humans [97].

Furthermore, changes in antibody production occur during ageing. It has been shown that splenic lymphocytes in older rats have a slower rate of capping and shedding of cross-linked surface receptors than those taken from young animals. The rate of capping can be increased in young



**Figure 7:** Changes in B-cell populations due to ageing. Figure taken from [94]

animals by the use of colchicine, which could not be proven for old animals. These functional changes lead to a lower rate of lymphocytes with high density of surface immunoglobulin in old rats compared to in young animals [98].

### 1.2.2.3 Age related changes in cytokine levels

As already mentioned in chapter 1.2.2.1, the levels of IL-6 and TNF- $\alpha$  produced by cells taken from old healthy donors are increased. In addition, also the levels of IL-1 $\beta$  were increased after stimulation [99]. There are indications that these increases in cytokines might be protective as lower levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-1Ra and IL-10 after stimulation in aged humans can be considered a marker for increased mortality risk. Subjects with low cytokine levels have been discovered to have twice the mortality risk compared to peers with higher levels of both pro- and anti-inflammatory cytokine levels. It has also been found that there is a genetic predisposition for expressing low cytokines that is associated with the IL-10 promoter gene. People with this predisposition have a greater risk to lose the capacity to respond to stimuli [100].

In contrast to the described increase in mortality risk due to decreased cytokine levels, it has been shown that mice that are unable to produce IL-6 have a decreased mortality when being stimulated with lipopolysaccharide in comparison to wild type animals [101]. In wild type animals, as described in the previous chapter, the levels of IL-6 were increased in old animals compared to young ones [101]. Contrary to the increase of IL-6 and TNF $\alpha$  described above, studies on humans have shown a defect in the induction of both IL-6 and TNF $\alpha$  [102] in healthy older test subjects. This has been explained to be due to a defect in toll like receptor 1/2. While other members of the toll like receptor family have been shown to be altered in aged mice, this could not be found in the human subjects [102]. These findings might also account for higher infection rates in older patients.

### 1.3 Animal Models

Even though COPD is a disease mainly witnessed in humans, experiments can of course not be conducted on human subjects as this would be in conflict with ethical guidelines. The conflict can be solved by using animal models as a substitute for humans. However not all animals are suited for such experiments. Some animals, such as rats are mostly insusceptible to cigarette smoke induced emphysema [103]. Contrariwise guinea pigs seem to be very susceptible to cigarette smoke induced emphysema [104],[105]. The problem with guinea pigs however is, that it would take a lot of living space and equipment to conduct experiments on them.

The model that is widely used and by now a standard model for simulating cigarette smoke induced COPD is the mouse model. Its advantages are the low costs for both the animals and the housing, as well as well established materials and equipment. On the other hand it is very important to find a strain of mice susceptible to cigarette smoke, as not all of them are. In the literature the C57BL6/J mouse line has been described to be one of the strains susceptible to cigarette smoke induced emphysema development [103], which is why these animals were used for the experiments. Mice have been shown to tolerate cigarette smoke exposure for at least a year, even though their activity seems to decrease. Mice only breathe through their nose, while humans smoke through their mouths, thus eliminating the clearing effect of the nose. In mice the clearing effect of nasal breathing seems less developed than in humans. This means that in mice even nasal breathing of cigarette smoke shows similar effects to human cigarette smoking [103]. Young mice have been shown to develop emphysematous changes in lung structure after 24 weeks of cigarette smoke exposure [106].

In humans women have been described to be more susceptible to cigarette smoke induced COPD, especially in the early stages of COPD development [19]. March and al. have discovered that an increased susceptibility in females also applies for most mouse strains [107]. For this reason we also used female animals for the experiments. Our experiment were conducted by the use of cigarette smoke in a smoke chamber. This method is the closest we can get to simulating human smoking habits. In contrast to a side stream model, exposure to mainstream cigarette smoke results in an inflammatory response, dominated by both macrophages and neutrophils, whereas the inflammation due to side-stream smoke is dominated by macrophages only. In addition, side-stream smoke has been shown to contain much higher levels of CO when compared to mainstream smoke of the same particle concentration [108]. Since CO has been shown to have a protective effect against lung inflammation, especially intra-alveolar neutrophil infiltration, and we aimed to examine the effects of inflammaging on COPD development, we decided against the sidestream smoke model [109].

### 1.4 Aim of Research

As the previous chapters have described in detail, COPD is a disease mainly caused by the body's inflammatory response to cigarette smoke. Also the changes in immune response occurring due to the ageing immune system have been described above. From the epidemiologic studies it is clear that the incidence of COPD rises with the age of the patients. The question now is whether ageing, in particular immunoageing, makes an organism more susceptible to cigarette smoke induced COPD, or whether this might only be the effect of cumulative exposure to cigarette smoke. Furthermore, the physiological decline in lung function with age, may also play a role in the high incidence of COPD amongst the old.

In earlier experiments using the same experimental set-up for cigarette smoke exposure it

has been found that young animals develop emphysema and lung function changes after four months of cigarette smoke exposure [81]. So, in order to determine whether old mice are indeed more susceptible to cigarette smoke induced COPD, the changes in lung function of older and younger animals exposed to cigarette smoke would be monitored to see if any differences could be determined at any of the time points. If older mice were to develop changes in these parameters earlier or more prominently compared to younger animals this would be indicative to a greater susceptibility to cigarette smoke. Such findings would then be the starting point for a more in depth analysis of the lungs to determine all the indicators of COPD as well as the cause of the greater susceptibility of aged mice to COPD development.





## 2 Methods and Materials

### 2.1 Animals & maintenance

Pathogen free female C57BL/6 mice aged 2 (8 to 10 weeks) and 12 months (56 weeks) were quartered in chambers in groups of four. They were exposed to a 12 hour light cycle with constant temperature between 23 and 25 degrees Celsius. Humidity levels were also kept constant. While in housing, the animals were exposed only to filtered air in each of the chambers. The mice were given unlimited access to food and water. Each of the four groups contained 8 animals. All experiments were conducted under strict governmental and international guidelines and were approved by the local government for the administrative region of Upper Bavaria.

### 2.2 Cigarette smoke exposure in a mouse model

The mice were exposed to 100 % mainstream cigarette smoke with a concentration of 500 mg/m<sup>3</sup> total particulate matter, to imitate human smoking habits. To generate mainstream cigarette smoke 3R4F cigarettes were used. A whole body exposure was performed by use of a smoke chamber. Exposure time was 50 minutes twice per day, five days per week for up to three months. After two months the first set of lung function tests were conducted which were repeated after 3 months of smoke exposure (see chapter ??). Control mice were handled, but only exposed to filtered air. 24 hours after the last cigarette smoke exposure the mice were sacrificed.

Total particulate matter was measured by using gravimetric analysis of quartz filters before and after air sampling from the smoke chamber and by measuring total air sample volume. To determine the amount of CO produced by cigarette smoking, CO concentrations were constantly measured using a GCO 100 CO meter with concentrations between  $288 \pm 74$  ppm. No toxic effects of CO exposure at these levels were witnessed in cigarette smoke exposed animals, CO-Hb levels were measured at  $12.2 \pm 2.4$  %.

Product	Company
Research Cigarettes 3R4F	Tobacco Research Insitute, University of Kentucky, Lexington, KY, USA
Whatman <sup>TM</sup> Glass microfibre filters Diameter 25 mm	GE Healthcare UK limited, Buckinghamshire, UK
GCO 100 CO Meter	Greisinger Electronic, Regenstauf, Germany

**Table 5:** Materials used for cigarette smoke experiments and measurements

## 2.3 Lung function analysis

Lung function analysis was performed using the flexiVent System (Scireq, Montreal, Canada) after two months and were repeated after three months of cigarette smoke exposure under anaesthesia with a ketamine-xylazine mixture using a non-tracheotomic intubation. The mice were ventilated with a tidal volume of 10 mL/kg with a frequency of 150 min<sup>-1</sup> to simulate a lung volume close to the one reached by physiological breathing. The mechanical properties, dynamic lung compliance and resistance, were determined by using a software generated script. Dynamic lung compliance was defined as  $C_{def} = \frac{V_T}{P_{IP} - P_{EEP}}$  with  $V_T$  being tidal volume,  $P_{IP}$  being peak inspiratory pressure and  $P_{EEP}$  being positive end-expiratory pressure. Changes in lung compliance occur in many diseases of the lung. Pulmonary fibrosis for example shows a decrease in lung compliance, whereas in COPD and lung emphysema the lung compliance increases. Airway resistance is defined as  $R_{AW} = \frac{\Delta P}{\Delta V}$ .  $\Delta p$  is the change in pressure, while  $\Delta V$  describes the following change in lung volume. Changes in airway resistance mostly occur when the diameter of the airways decreases, as it is the case in COPD patients. To get a more precise value these measurements were determined four times for each animal.

## 2.4 Bronchioalveolar lavage (BAL) sampling and processing

Product	Company
PBS	Gibco, Life Technologies, Darmstadt, Germany
RPMI-1640	Gibco, Life Technologies, Darmstadt, Germany
FCS	Gibco, Life Technologies, Darmstadt, Germany
Objektträger	

**Table 6:** Materials used for preparation of BAL

Bronchioalveolar lavage was performed by using four aliquots of 0,5 mL sterile PBS for intra-tracheal instillation into the lungs. For the preparation of cytopins the lavaged cells were spun down at 400 g. Afterwards cells were re-suspended in RMPI- 1640 medium in a solution containing 10% FCS. Total cell counts were then determined using a hemocytometer. Afterwards 30 000 cells per sample were spun down onto histological slides. These slides were then stained using the May-Grünwald-Giemsa staining as described in chapter 2.7.1. Differential cell counts of 200 cells per sample on these slides were performed using morphological criteria. BAL fluids were used to determine cytokine secretion via multiplex analysis.

## 2.5 Tissue preparation

24 hours after the last cigarette smoke exposure mice were sacrificed. In four animals of each group the left lung was fixed by intra-tracheal instillation of 6% para-formaldehyde (PFA) buffered with PBS with a constant pressure of 20 cm fluid column. The tissue was then embedded in 2% agar solution to ensure the best stereological analysis possible. After hardening of the agar around the tissue the samples were dehydrated using a Leica Tp 1020 embedding machine. Dehydration was performed using alcohol solutions with increasing concentrations

Product	Company
PBS	Gibco, Life Technologies, Darmstadt, Germany
Para-formaldehyde	SigmaAldrich, St. Louis, MO, USA
Agar	SigmaAldrich, St. Louis, MO, USA
Paraffin	
Thermo Scientific super-frost plus Microscope slides	Gerhard-Menzel GmbH, Braunschweig, Germany
Feather Microtome blade S35	Feather Safety Razor Co. LTD, Osaka, Japan

**Table 7:** Materials used for tissue preparation and cutting histological slides

(starting at 70% and increasing to 80%, 96% for a few times and then increasing to 100%) followed by suspension in xylene. Afterwards the tissue was embedded in paraffin blocks for cutting. The blocks were then cut using the Zeiss Microtome with Feather blades to 3  $\mu$ m thickness and mounted onto slides. The the right lung was shock frozen in liquid nitrogen and then stored in -80°C for isolation of RNA and protein as described in chapter 2.9. The lungs of the remaining four animals per group were used for FACS analysis as described in chapter 2.6. In addition to the preparation of the lung, spleens from all the groups were harvested for FACS analysis (see chapter 2.6).

## 2.6 Fluorescence-activated cell sorting (FACS)

For the determination of lymphocyte infiltration and analysis of subtypes whole, lung tissue prepared as described in chapter 2.5 was used. Lungs were first perfused with sterile PBS through the right ventricle to clear leukocytes and erythrocytes from lung circulation in order to only analyse lung tissue. Lung homogenisation was then performed by applying lung dissociation buffer for enzymatic digestion. Mechanical dissociation of the tissue was then performed by use of the gentleMACS dissociator (Miltenyi Biotec). Afterwards samples were filtered to remove large particles remaining from dissociation from the single cell suspensions. Cells were afterwards blocked using CD16/CD32 antibodies.

The samples were split and stained for different analyses. One part of the sample was stained with the Mouse Regulatory T-cell Staining Kit which uses an anti-FoxP3 antibody to determine the amount of regulatory T-cells in lung tissue. The other part of the sample was centrifugated using Pancoll for density gradient centrifugation. These samples were then used for the isolation of mono-nuclear cells, which were then cultured over night on Anti-CD3/ Anti-CD28 coated plates to perform intracellular cytokine staining. Restimulation with a leukocyte activation cocktail with Golgi plug for four hours was performed the following day. Cells were afterwards stained with anti-CD4 antibody and fixed with 2% formaldehyde solution and permeabilized with 0.3% saponin buffer for intracellular staining. To distinguish the different T-helper cell sub-populations the cells were stained with antibodies against IL-17A, IFN $\gamma$  and IL-4. Differentiation was performed using multicolour analysis of the stained cells with a BD FACSCanto II flow cytometer and BD FACSDiva software.

Spleens sampled as described in chapter 2.5 were homogenized mechanically by using the gentleMACS Dissociator and afterwards filtered to remove larger particles and obtain a single

Product	Company
PBS	Gibco, Life Technologies, Darmstadt, Germany
Lung Dissociation Buffer	Miltenyi Biotec, Bergisch Gladbach, Germany
Gentle MACS Dissociator	Miltenyi Biotec, Bergisch Gladbach, Germany
Mouse Regulatory T Cell Staining Kit	eBioscience, San Diego, CA, USA
Pancoll	PAN Biotech, Aidenbach, Germany
Anti-CD3/ Anti CD28 coated plates	
Golgi Plug	BD Pharmingen, San. Diego, CA, USA
Formaldehyde	Aldrich Chemistry, St. Louis, MO, USA
0.5% Saponin Buffer	Sigma Aldrich, St. Louis, MO, USA
Anti- Mouse Anti-CD4 Antibody	eBioscience, San Diego, CA, USA
Anti- Mouse Anti-IL17A Antibody	eBioscience, San Diego, CA, USA
Anti- Mouse Anti-IFN Antibody	eBioscience, San Diego, CA, USA
Anti- Mouse Anti-IL4 Antibody	Biozol, Eching, Germany
Anti- Mouse Anti-CD16/CD32 Antibody	Biozol, Eching, Germany
Anti- Mouse Anti-CD44 Antibody	eBioscience, San Diego, CA, USA
Anti- Mouse Anti-CD69 Antibody	eBioscience, San Diego, CA, USA
Anti- Mouse Anti-CD62L Antibody	eBioscience, San Diego, CA, USA
BD FACSCanto II Flow Cytometer	BD Biosciences, Heidelberg, Germany

**Table 8:** Materials used for FACS-analysis including antibodies

cell suspension. Splenocytes were blocked by using CD16/ CD32 antibodies before being stained with anti-mouse anti-CD4, anti-CD44, anti-CD69 and anti-CD62L antibodies. The stained cells were afterwards analysed by using a BD FACSCanto II flow cytometer running BD FACSDiva software.

## 2.7 Histological staining

### 2.7.1 May-Grünwald-Giemsa staining

Slides prepared as described in chapter 2.4 were dried over night at 38 °C and stained the next day. After labelling the slides, they were suspended in May-Grünwald's eosine-methylene blue solution for 10 minutes. Afterwards the slides were be rinsed in tap-water. The next step was suspending the slides in Giemsa's Azur-eosine methylene blue solution, diluted 1:20 using tap water, for 15 minutes. Afterwards slides were rinsed in tap water again and dried off at room

Product	Company
May-Grünwald's eosine-methylene blue solution modified	Merck, Darmstadt, Germany
Giemsa Azur-Eosine methylene blue solution	Merck, Darmstadt Germany
Entellan New	Merck, Darmstadt, Germany
Coverslips 18×18 mm Thickness No. 1	VWR, Darmstadt, Germany

**Table 9:** Materials used for May-Grünwald-Giemsa staining

temperature. To protect the stained tissue, the slides were covered using Entellan mounting medium and cover slides.

### 2.7.2 Haematoxylin-Eosin staining

Product	Company
Eosin yellowish	Merck, Darmstadt, Germany
Mayer's Hemalaun	Merck, Darmstadt Germany
Hydrochloric acid (HCl)	Merck, Darmstadt, Germany
Xylene	Roth, Karlsruhe, Germany
Entellan New	Merck, Darmstadt, Germany
Coverslips 24×50 mm Thickness No. 1	VWR, Darmstadt, Germany

**Table 10:** Materials used for HE staining

The slides (prepared as described in chapter 2.5) were stored over night at 38°C before staining. For staining the tissue, it was necessary to remove the paraffin first. To this end the slides were first deparaffinized by suspending them in pure xylene for two times 5 minutes. As the staining was performed in hydrophilic solutions the tissue needed to be rehydrated. Rehydration was achieved by suspending the slides in solutions of ethanol with decreasing concentrations (100%, 100%, 90%, 80%, 70% 1 minute in each solution) and then rinsed in distilled water. Afterwards the nuclei of the cells were stained using Mayer's Haemalaun solution for 5 minutes and afterwards differentiated using a solution of 1% HCl-ethanol for 2 minutes. After rinsing the slides in both tap-water and distilled water, the slides were stained with a ready to use eosin solution for 8 minutes. Since the preservation of the tissue was done with a xylene based substance, a dehydration of the tissue was necessary. This was achieved by placing the slides in solutions of ethanol with increasing concentrations (70%, 80%, 90%, 96%, 100%, 100% each for 1 minute). Afterwards slides were placed in pure xylene for two times 5 minutes. To protect the tissue, cover-slips were then mounted using Entellan.

### 2.7.3 Trichrome staining

Slides were prepared as described in chapter 2.5 and stored over night at 38°C. as described before, the tissue needed to be deparaffinated before staining. This was achieved by suspending

Product	Company
Weigert Iron Hematoxylin A+B	Merck, Darmstadt, Germany
Ponceau 2R	Sigma Aldrich, St. Louis, MO, USA
Acid fuchsin	Merck, Darmstadt, Germany
Acetic Acid	Merck, Darmstadt, Germany
Phosphomolybdic acid	Merck, Darmstadt, Germany
Orange G	Merck, Darmstadt, Germany
Light Green SF	Merck, Darmstadt, Germany
Xylene	Roth, Karlsruhe, Germany
Entellan New	Merck, Darmstadt, Germany
Coverslips 24×50 mm Thickness No. 1	VWR, Darmstadt, Germany

**Table 11:** Materials used for Masson's-Trichrom staining

the slides in pure xylene for two times 5 minutes. Afterwards rehydration of the tissue was performed by placing the slides in solutions of ethanol with decreasing concentrations (100%, 100%, 90%, 80%, 70% each for 1 minute). Afterwards slides were directly placed into ready to use Weigert's iron hematoxylin solution for 4 minutes. Differentiation was performed in 1% HCl-ethanol solution. The slides were then rinsed in tap water. Afterwards slides were suspended in Ponceau-acid-fuchsin solution for 5 minutes and then washed in a solution of 1% acetic acid. In the next step slides were stained with phosphomolybdic acid- orange G solution for 7 minutes and again washed in 1% acetic acid solution. The last staining was performed with light green solution for 10 minutes. Afterwards the slides were to be washed in distilled water and then dehydrated using ethanol solutions with increasing concentration (70%, 80%, 90%, 96%, 100%, 100% each for 1 minute). After suspending the slides in pure xylene for two times 5 minutes, coverslips were mounted with Entellan.

### 2.7.4 Immunohistochemistry

Immunohistochemical staining of the lung tissue was also performed. Slices were cut to 3  $\mu$ m thickness as described in chapter 2.5 and stored at 58°C over night. For deparaffinization of the tissue, the slides were suspended in pure xylene for two times 5 minutes. Afterwards rehydration was necessary because all the staining solutions are hydrophilic. This was achieved by putting the slides in solutions of ethanol with decreasing concentration (100%, 100%, 90%, 80%, 70%, each for 1 minute). The slides were then rinsed in distilled water. Endogenous peroxidase activity was then blocked by placing the slides in a solution of 1.8% H<sub>2</sub>O<sub>2</sub> (in 80% methanol and 20% distilled water) for 20 minutes. A heat induced epitope retrieval was performed in ready to use HIER Citrate Buffer, diluted as given by the manufacturer, in a De-cloaking chamber. The slides were first heated up to 125 °C and then allowed to cool down to 90 °C. After removing the container of slides from the de-cloaking chamber, they were left to cool down for another ten minutes at room temperature. Afterwards the slides were further cooled down by exchanging half of the hot citrate buffer with room temperature Rockland buffer. This is repeated four times. To prevent unspecific binding of antibodies, tissue blocking was performed using rodent blocking antibody for 30 minutes. The slides were then washed in Wash

Product	Company
Xylene	Roth, Karlsruhe, Germany
Hydrogenperoxide (H <sub>2</sub> O <sub>2</sub> ) solution	Sigma Aldrich, St. Louis, MO, USA
HIER Citrate Buffer pH 6.0 ZUC 028	Zytomed Systems, Berlin, Germany
Decloaking chamber	Biocare Medical, Concord, CA, USA
Rodent Block M	Biocare Medical, Concord, CA, USA
Rockland 10× TBS buffer pH 7,5	Rockland, Limerick, PA, USA
TRIS Wash Buffer TBS 20× ZUC 066	Zytomed Sytems, Berlin, Germany
Rabbit On Rodent AP-Polymer	Biocare Medical, Concord, CA, USA
Rat on Mouse AP-Polymer	Biocare Medical, Concord, CA, USA
Vulcan Fast Red Chromogen Kit	Biocare Medical, Concord, CA, USA
Haematoxylin	Sigma-Aldrich St. Louis, MO, USA

**Table 12:** Materials used for immunohistochemistry

Target	Dilution	Origin	Company
MMP12	1:400	rabbit	Abcam, Cambridge, UK
CD3	1:300	rabbit	Sigma Aldrich, St. Louis, MO, USA
CD21	1:100	rabbit	Novus Biologicals, Littleton, CO, USA
CD45R/B220	1:50	rat	BD Pharmingen, San Jose, CA, USA
IL17	1:100	rabbit	Santa Cruz Biotechnology, Dallas, Texas, USA
P16	1:50	rabbit	Santa Cruz Biotechnology, Dallas, Texas, USA
SIRT1	1:100	rabbit	Merck Millipore, Darmstadt, Germany
β-Galactosidase	1:150	rabbit	life Technologies, Eugene, OR, USA

**Table 13:** List of antibodies used for immunohistochemistry

buffer (ZUC 066) by placing them in the buffer for 2 minutes each in 3 different containers. Afterwards tissue was incubated with primary antibody, diluted as given in table 13 with antibody diluent, for at least 8 hours at 4°C. After incubation with the primary antibody, slides were washed in ZUC66 buffer as described above. Afterwards they were incubated with an alkaline phosphatase labelled secondary antibody directed at the primary antibody (Rabbit On Rodent, Biocare Medical or Rat on Mouse, Biocare) for 30 minutes. Again slides were washed with ZUC66 buffer. Afterwards signals were intensified using chromogen substrate Vulcan Fast Red, prepared as given by the manufacturer and incubated for 7 to 12 minutes. After washing in ZUC66 buffer and rinsing in distilled water, slides were counter-stained with a ready to use hematoxylin solution for three minutes. Afterwards the slides needed to be washed again and were placed in the buffer for 5 minutes to drain excess colour. As the slides were covered using a xylene based mounting medium, the tissue needed to be dehydrated. This was performed by placing the slides in solutions of ethanol with increasing concentrations (96%, 96%, 100%, 100% for 1 minute each) and then placed in pure xylene for two times 5 minutes. Slides were then protected by mounting cover-slips with a Entellan.

## 2.8 Stereological analysis

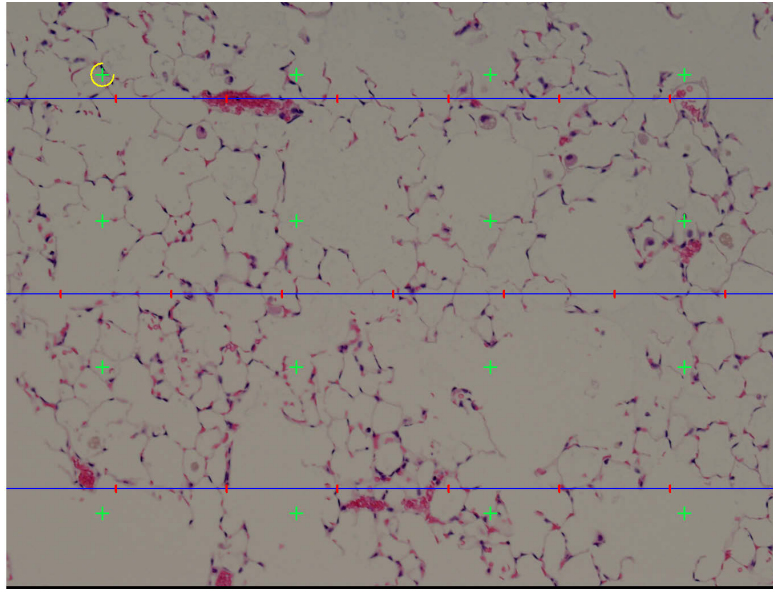
Stereological analysis was performed on tissue of the left lung of the mice. Slides were analysed using an Olympus BX51 light microscope equipped with a computer-assisted stereological toolbox (NewCAST, Visiopharm, Hoersholm, Denmark).

### 2.8.1 Mean Chord Length

Emphysema development was quantified by measuring the mean linear intercept or mean chord length (MCL) and alveolar surface area in HE stained slides (prepared as described in chapters 2.5 and 2.7.2). Measurements were performed on 30 random fields of view with  $20\times$  magnification using a superimposed grid (see Figure 8). Intercepts of the blue lines with alveolar septa as well as any of the red ticks falling on alveolar airspace were counted for the calculation of MCL as follows:

$$MCL = \frac{2 \cdot \Sigma P_{air} \cdot L(p)}{\Sigma I_{septa}} [\mu m] \quad (2.1)$$

Red ticks of the grid hitting alveolar airspace are labeled  $P_{air}$ , length per point  $L(p)$  is the distance between two red ticks, while the intersections of the grid with alveolar walls are labeled  $I_{septa}$ . In equation 2.1 the sum is taken over all fields of view.



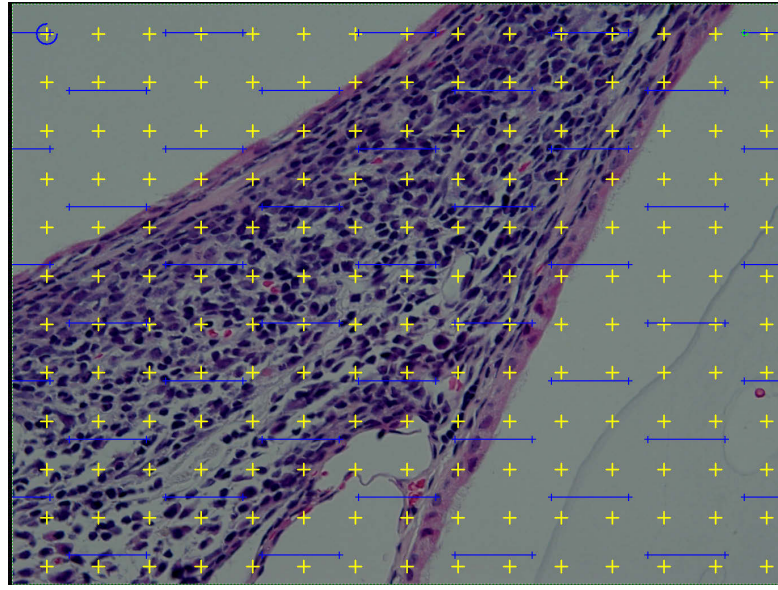
**Figure 8:** Screen-shot of newCAST grid for MCL counting, Blue lines for intercept with alveolar septa, red ticks for quantification of air space



### 2.8.2 Inducible Bronchus Associated Lymphoid Tissue

A stereological analysis of the volume of inducible bronchus associated lymphoid tissue (iBALT) was performed. For quantification a different grid was superimposed on 100 fields of view of HE stained lung tissue with a magnification of  $40 \times$  (see Figure 9). Points (yellow) falling on iBALT structures were counted as  $P_{iBALT}$  to determine iBALT volume, while lines intersecting with basal membrane of either airways or vessel were defined as  $I_{airway+vessel}$  to quantify basal membrane area. The amount of iBALT structures in relation to basal membrane area was defined as:

$$\left(\frac{V}{S}\right)_{iBALT} = \frac{\Sigma P_{iBALT} \cdot L(p)}{\Sigma I_{airway+vessel}} \left[ \frac{\mu m^3}{\mu m^2} \right] \quad (2.2)$$

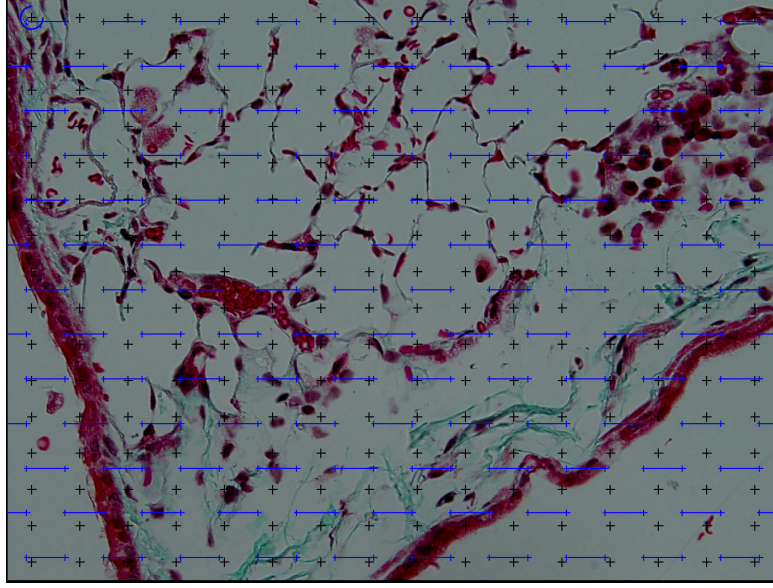


**Figure 9:** Screen-shot of newCAST grid for iBALT counting, lines for intercept with basal membrane of either vessel or airway, points for counting of iBALT volume

### 2.8.3 Airway Remodelling

Furthermore, counting of collagen fibres was conducted to quantify the degree of airway remodelling. Counting was performed on lung tissue prepared as described in chapter 2.5 and stained as described in chapter 2.7.3. Again a special grid was superimposed on 100 fields of view with a magnification of  $40 \times$  (Figure 10). Collagen was counted only around airways, not around vessels, these points being defined as  $P_{collagen}$ . Lines intersecting with either airway or vessel basal membrane were defined as  $I_{airway+vessel}$ .

$$\left(\frac{V}{S}\right)_{Collagen} = \frac{\Sigma P_{Collagen} \cdot L(p)}{\Sigma I_{airway+vessel}} \left[ \frac{\mu m^3}{\mu m^2} \right] \quad (2.3)$$



**Figure 10:** Screen-shot of newCAST grid for quantification of airway remodelling, lines for counting of intersections with basal membrane of vessels and airways, points for quantification of collagen fibre volume

## 2.9 cDNA Transcription and Quantitative Real Time PCR

Frozen tissue of the right lung was homogenised by use of Micro-Dismembrator-S for 1 min at  $3000 \text{ min}^{-1}$ . Homogenised tissue was then dissolved in Roti-Quick 1 Solution. After adding Roti-Quick 2 Solution and extracting RNA from the homogenate further extraction was performed using the peqGOLD Total RNA Kit according to the instructions given in the kit. Synthesis of cDNA was performed using Random Hexamers and MuL V Reverse Transcriptase.

Expression of target genes (see table 14) was determined in comparison to housekeeping control hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT-1) using Platinum SYBR Green qPCR SuperMix and StepOnePlus<sup>TM</sup> 96 well Real-Time PCR System (Applied Biosystems). Primers (see table 14) were designed by the use of Primer-BLAST software according to published mRNA sequences.

Relative transcript expression of genes is defined as  $2^{-\Delta Ct} = 2^{Ct_{\text{target}} - Ct_{\text{reference}}}$ , comparison to control group was defined as  $2^{\Delta\Delta Ct} = 2^{\Delta Ct_{\text{treated}} - \Delta Ct_{\text{control}}}$

Gene	Forward Primer	Reverse Primer
CXCL 13	TCT CTC CAG GCC AGG CAT TTC T	ACC ATT TGG CAC GAC GAT TCA C
F4/80	CTC TGT GGT CCC ACC TTC AT	GAT GGC CAA GGA TCT GAA AA
Foxp3	AGA GCC CTC ACA ACC AGC TA	CCA GAT GTT GTG GGT GAG TG
Gata 3	GTC ATC CCT GAG CCA CAT CT	TAG AAG GGG TCG GAG GAA CT
HPRT 1	CCT AAG ATG AGC GCA AGT TGA A	CCA CAG GAC TAG AAC ACC TGC TAA
IFN	TCC AGA GCT GCA GTG ACC	GAA GCA CCA GGT GTC AAG
IL-6	GTT CTC TGG GAA ATC GTG GA	TGT TCA CCA GGT AGC TAT GG
IL-13	TAC GGT CTC CAG CCT CCC CG	GGC CGT GGC GAA ACA GGT GC
KC (CXCL 1)	CCG AAG TCA TAG CCA CAC	GTG CCA TCA GAG CAG TCT
MIP1	CAC CAT ATG GCT CGG ACA CC	TCA GGA AAA TGA CAC CTG GCT
MMP12	TGT ACC CCA CCT ACA GAT ACC TTA	CCA TAG AGG GAC TGA ATG TTA CGT
p16	TCG TGA ACA TGT TGT TGA GGC	CTA CGT GAA CGT TGC CCA TC
p21	CGG TGT CAG AGT CTA GGG GA	AGA GAC AAC GGC ACA CTT TG
Roryt	CAA GTC ATC TGG GAT CCA CTA C	TGC AGG AGT AGG CCA CAT TAC A
SIRT1	CCA TTA ATG AGG AAA GCA ATA GGC	AAT ACA AGG CTA ACA CCT TGG G
TNF- $\alpha$	CAC CAC GCT CTT CTG TCT	GGC TAC AGG CTT CTG ACT C
Muc5AC	ATC GAG AGG ACG GTT GAC AC	ATC CAG CCT TGC TTG

**Table 14:** List of primers for quantitative real time PCR

Product	Company
Micro-Dismembrator-S	Sartorius, Göttingen, Germany
peqGOLD Total RNA Kit	Peqlab, Erlangen, Germany
Roti Quick Kit	Roth, Karlsruhe, Germany
MuL V Reverse Transcriptase	Applied Biosystems, Darmstadt, Germany
Platinum SYBR Green qPCR SuperMix	Applied Biosystems, Carlsbad, CA, USA
Random Hexamers	Applied Biosystems, Carlsbad, CA, USA
StepOnePlus™ 96 well Real-Time PCR System	Applied Biosystems, Carlsbad, CA, USA

**Table 15:** Materials for tissue homogenisation, isolation of RNA, production of cDNA and quantitative real time PCR

## 2.10 Protein isolation and Western Blot

The shock frozen lung tissue prepared as described in chapter 2.5 was homogenized as described in chapter 2.9. A small portion of the still frozen lung tissue powder was then dissolved in RIPA-Buffer (prepared as described in table 17). After thorough mixing, the solution was centrifuged and the supernatant taken for further analysis.

### 2.10.1 Protein concentration analysis

Protein concentration was then determined using the BCA assay kit, in comparison to a standard curve with known concentrations of bovine serum albumin ranging from 2 mg/mL to a blank that only contained RIPA buffer.

Protein probes were diluted 1:10 in RIPA buffer for more accurate analysis. For each of the probes 25  $\mu$ L of the diluted protein solution were taken into micro-plate wells with 200  $\mu$ L of

Product	Company
Albumine from bovine serum (BSA)	SigmaAldrich, St. Louis, MO, USA
Laemmli sample buffer	BioRad Laboratories, Hercules, CA, USA
Rotiphorese Gel 30 (37,5:1)	Roth, Karlsruhe, Germany
SDS Solution 10%	AppliChem, Darmstadt, Germany
BioRad Precision Plus Protein Dual Color Standards	BioRad Laboratories, Hercules, CA, USA
TEMED 20	BioRad Laboratories, Hercules, CA, USA
Milkpowder, Blotting Grade	Roth, Karlsruhe, Germany
ECL Prime Western Blotting Detection Reagent	GE Healthcare, Munich, Germany
Immun-Blot PVDF Membrane for Protein Blotting	BioRad Laboratories, Hercules, CA, USA

**Table 16:** Materials used for protein isolation and western blot

Ingredient	Concentration
Sodiumchloride (NaCl)	150 mM
Tris	10 mM pH 7.2
SDS	0.1%
Triton X-100	1%
Deoxycholat	1%
EDTA	5 mM
Complete Protease Inhibitor	1:25
Vandate	1:100

**Table 17:** RIPA buffer recipe and ingredients

Ingredient	Amount
Tris	15.1 g
SDS	50 mL
Glycin	94 g
Distilled water	900 ml

**Table 18:** Electrophoresis buffer recipe to be diluted 1:5 in distilled water and ingredients

Ingredient	Amount
Methanol	200 mL
Tris	2.42 g
Glycin	11.2 g
Distilled water	800 mL

**Table 19:** Transfer buffer recipe

working reagent added for analysis. The plate was then incubated for 45 min at a temperature of 37 °C before analysis.

### 2.10.2 Western Blot

In preparation for the western blot analysis, gels were prepared the previous day to ensure complete polymerisation of the gel. The gels were prepared using BioRad glass plates with a thickness of 1,5 mm (the lower part consisting of separation gel and the upper part consisting of collection gel) and 15 wells for protein solution.

The following day gels were washed and placed in running buffer prepared as described in table 18 and afterwards the wells were loaded with the protein solution prepared as described in chapter 2.10.1. The first well of each gel was filled with marker protein. The gels were run with 400 mA and 100 V for 10 min to pass the protein through the collection gel and afterwards for then run for approximately 60 min to be separated.

After separation, the protein was transferred onto the membrane, that had been activated in methanol and placed onto the gel. The transfer was performed in cooled transfer buffer (see table 19) with 400 mA and 100 V for about 70-85 min. The membrane was afterwards washed in PBST and blocked in a solution of 5% milk (5 g milk-powder diluted in 100 mL of PBST). The membrane was again washed in PBST and incubated over night at 4°C with the primary antibody that was diluted according to table 21 in 1% milk solution.

After incubation for at least 8 hours, the membrane was washed and incubated with the secondary antibody diluted in 1% milk solution for an hour at room temperature. After another round of washing with PBST, the membrane was covered with blotting reagent and pictures were taken as described in chapter 2.10.3.

For the determination of  $\beta$ -actin the membrane was washed and stripped by incubating with a ready to use stripping buffer for 15 minutes at room temperature, washed again and then blocked in 5% milk solution for 30-60 min. After blocking the membrane was incubated with  $\beta$ -actin antibody diluted in 1% milk solution for an hour at room temperature. Afterwards the membrane was washed and then developed using the Amersham ECL detection reagent and photographed (see chapter 2.10.3).

### 2.10.3 Analysis of Western Blot

For the analysis of the western blot membranes, pictures were first taken with a ChemiDoc<sup>TM</sup> XRS+ with Image Lab<sup>TM</sup> Software. The chemiluminescent pictures were taken with different exposure times depending on the antibody used. To also be able to determine the size of the protein bands, an image under white light of the markers was taken and merged with the luminescence image. Analysis was then using the same software to quantify the intensity of

Ingredient	CG	SG	Company
Distilled Water	5.6 mL	5.8 mL	
Tris Buffer 1,5 M, pH 8.8	4.2 mL		
Tris Buffer 0,5 M, pH 6.8		2.5 mL	
Rotiphorese Gel 30 (37,5:1)	6.6 mL	1.7 mL	Roth, Karlsruhe, Germany
SDS	165 $\mu$ L	100 $\mu$ L	AppliChem, Darmstadt, Germany
TEMED 20	22 $\mu$ L	20 $\mu$ L	BioRad Laboratories, Munich, Germany
Ammoniumpersulfat (APS)	22 $\mu$ L	20 $\mu$ L	Sigma-Aldrich, St. Louis, MO, USA

**Table 20:** Separation (SG) and collection gel (CG) recipes and ingredients

Target	Dilution	Origin	Company
SIRT1	1:1000	rabbit	Millipore tenacula, CA, USA
p16	1:200	rabbit	Santa Cruz Biotechnology, Dallas, Texas, USA
p21	1:200	rabbit	Santa Cruz Biotechnology, Dallas, Texas, USA
p53	1:200	mouse	Santa Cruz Biotechnology, Dallas, Texas, USA

**Table 21:** List of antibodies used for Western blot

the luminescence bands, which was then compared to the luminescence emitted by the  $\beta$ -actin band on the same membrane after stripping.

## 2.11 Data analysis

For all the data analyses GraphPad Prism 6 software (GraphPad Software, La Jolla, USA) was used. The results are given as mean values, while the error bars indicate the standard deviation. To determine the significance of the results, One Way ANOVA tests were used to compare multiple groups. For One Way ANOVA, as for most statistical analyses, Gaussian distribution is required, so tests for normality were performed.

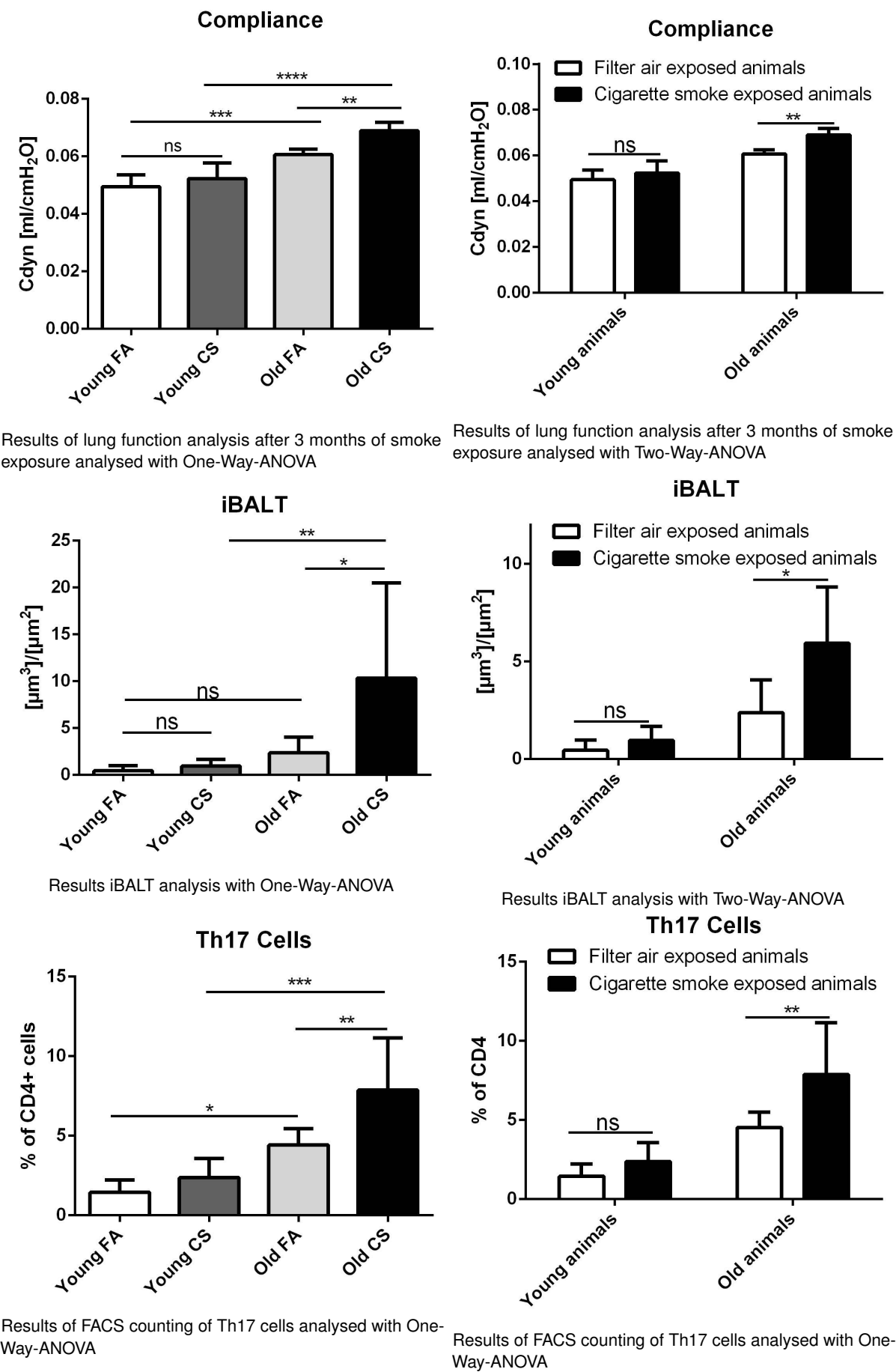
For some of our experiments we had to distribute the mice into two groups, because there was not enough tissue to perform all analyses on all mice. Therefore, for some analyses the number of values (N) was very small. This of course limits the credibility of a statistical analysis. The test for Gaussian distribution with the applied software package requires a minimum number of N= 8. Thus, for most of our tests, normal distribution could not be tested. We nevertheless assumed Gaussian distribution in all our analyses, since it was proven for all the analyses that had a number of N large enough to carry out the test. As lung function analysis was performed on all animals undergoing the experiment, the tests for Gaussian distribution have been performed on the lung compliance measurement. With a p-value ranging from 0.4 to 0.84 after two months of smoke exposure, the test was positive such that a Gaussian distribution can be assumed, or at least could not be disproved.

Although the number of test subjects in our experiment was small, we still reached significant results in most of the analyses performed.

In the figures presenting the results significant differences are marked with stars, the number of which indicate the p values of the statistical analysis used to determine significance. Any p value greater than 0.05 was considered not significant (ns).

ns	$p \geq 0.05$
*	$p \leq 0.05$
**	$p \leq 0.01$
***	$p \leq 0.005$
****	$p \leq 0.001$

As the experiment was designed with two independent variables, one being the age of the mice and the other being the exposition to cigarette smoke, a Two-Way-ANOVA analysis for the statistics of the data might be considered an optimal solution. To show that the results are quite robust independent of the applied method, for some of the data Two-Way-ANOVAs were performed additionally. The data sheets are presented below. For all other results, One-way-ANOVA was performed as presented in chapter 3.



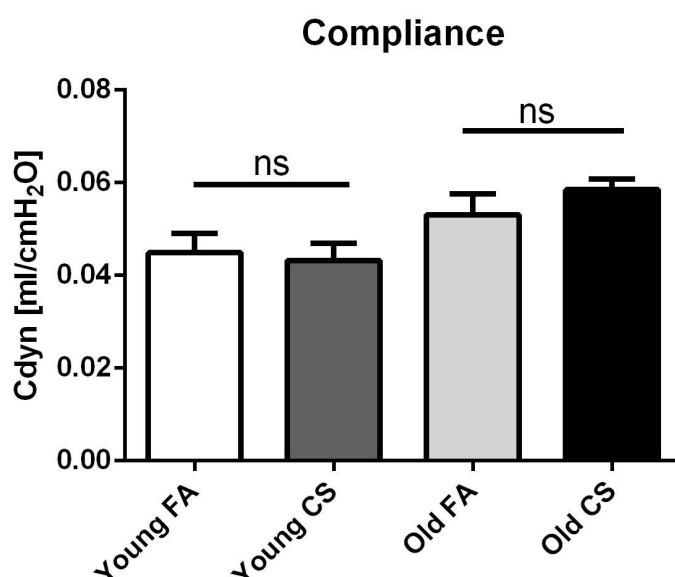
**Figure 11:** Comparison between the results of analysis with One-Way-ANOVA and Two-Way-ANOVA



## 3 Results

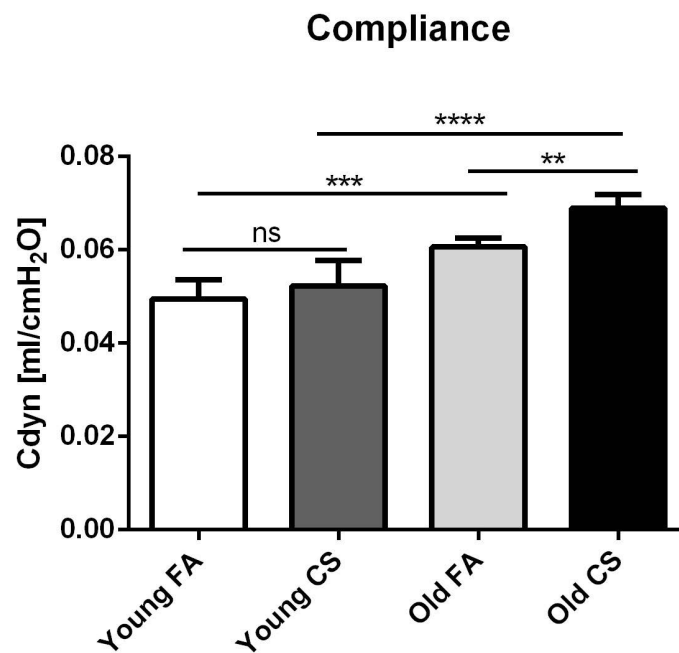
### 3.1 Lung Function Analysis

After two months of cigarette smoke exposure, lung function analysis was performed, as described in chapter 2.3. The objective was to investigate whether any differences in lung function between the groups occur due to cigarette smoke exposure. The most important markers of COPD development that can be determined in mice are lung compliance and airway resistance [110]. Accordingly these two markers were examined to determine the effects of cigarette smoke exposure. The data determined after two months of cigarette smoke exposure suggested no changes in lung function due to cigarette smoke exposure in either the young mice, or the old animals. However the baseline compliance in old, filter air exposed animals was significantly higher compared to the younger peer group (figure 12).



**Figure 12:** Lung compliance analysis after two months of smoke exposure: No significant changes between either the groups of young mice or the groups of old mice. The lung compliance of old mice is larger than that of the young animals.

As we had used a non-fatal method of intubation for this lung function test, we were able to continue the smoking experiments using the same mice for another month. After a total of three months of smoke exposure, the lung function tests were repeated. In the analysis of lung compliance, a significant increase for the old animals was determined, as can be seen in figure 13. Even though changes in lung function parameters could be determined in the old mice at this time-point, there still were no significant changes in the lung compliance of young animals due to cigarette smoke exposure. As has already been found after two months of cigarette smoke exposure, the baseline compliance for the old filter air exposed (FA) animals was increased compared to young FA animals (figure 13).



**Figure 13:** Lung compliance analysis after 3 months of cigarette smoke exposure. A significant increase in lung compliance in old animals due to cigarette smoke can be seen while there was no increase in lung compliance in young animals.

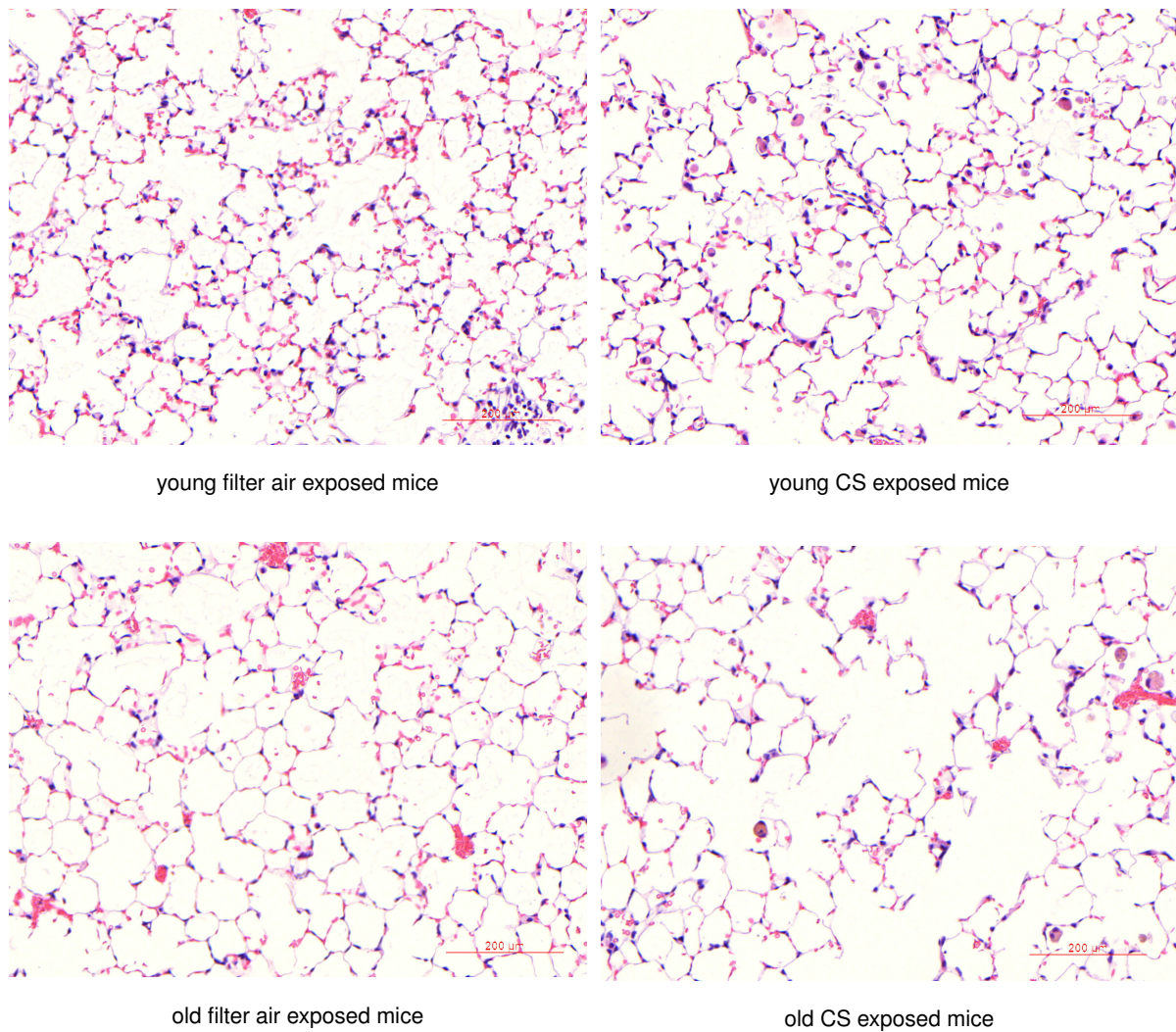
## 3.2 Stereological Analysis

### 3.2.1 Mean Chord Length

As emphysema development is one of the most important markers for the development of COPD, its quantification is very important to monitor the progress of the disease in our cigarette smoke mouse model. Changes between the groups are obvious from the HE-stained tissue (Figure 14). Quantification was performed as described in chapter 2.8.1. As shown in Figure 15, a significant increase in the mean chord length has been found already in the filter-air-exposed (FA) old mice compared to FA young animals, while no significant changes were observed in the young filter air exposed mice (white bars) compared to young mice with CS-exposure (dark grey bars). Most importantly, our analysis showed that there was a significant increase in the mean chord length in old CS-exposed animals (black bars) in comparison with the FA-exposed animals of the same age group (light grey bars) as well as in comparison with the CS-exposed animals in the group of young mice.

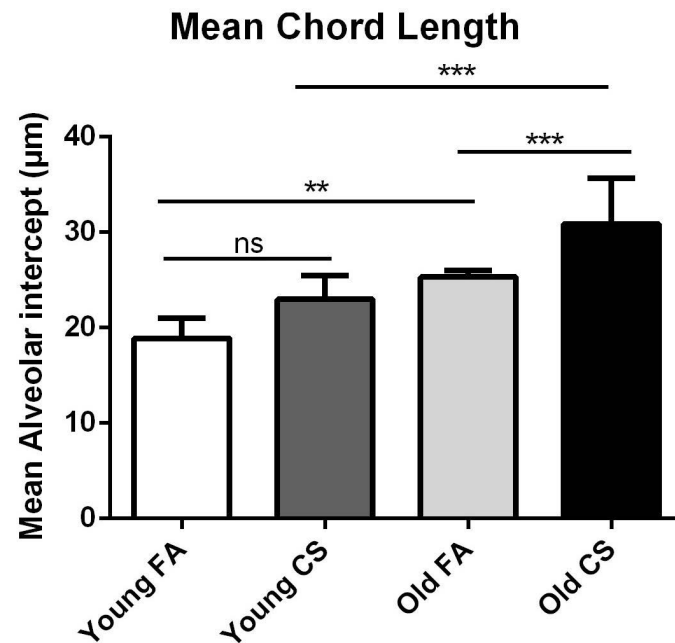
### 3.2.2 Airway Remodelling

Another mechanism in the development of COPD is small airway remodelling, which can be quantified by the increase in collagen fibres around the airways. Airway remodelling is partly responsible for the obstructive component in the COPD development, as the increase in collagen fibres is taking part in the decrease in airway area. As visible in figure 17, with lung tissue stained as described in chapter 2.7.3. Here an increase in the green stained collagen fibres in the airway walls can be found only in the old cigarette smoke exposed animals. Further analysis

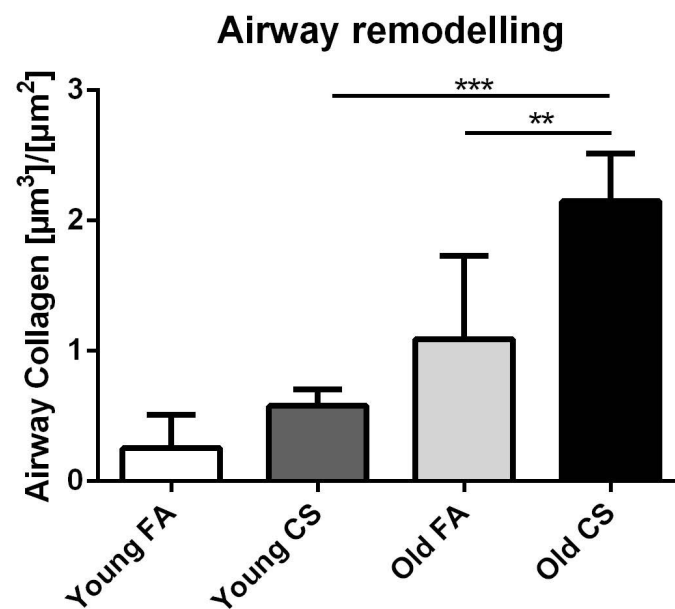


**Figure 14:** Lung tissue of the mice described in chapter 2.1 stained with HE as detailed in chapter 2.7.2. For the young animals no changes in airspace volume due to cigarette smoke exposure are visible. The old animals exhibit an increased airspace already in the filtered air exposed groups. Airspace enlargement increases due to cigarette smoke exposure in only old animals.

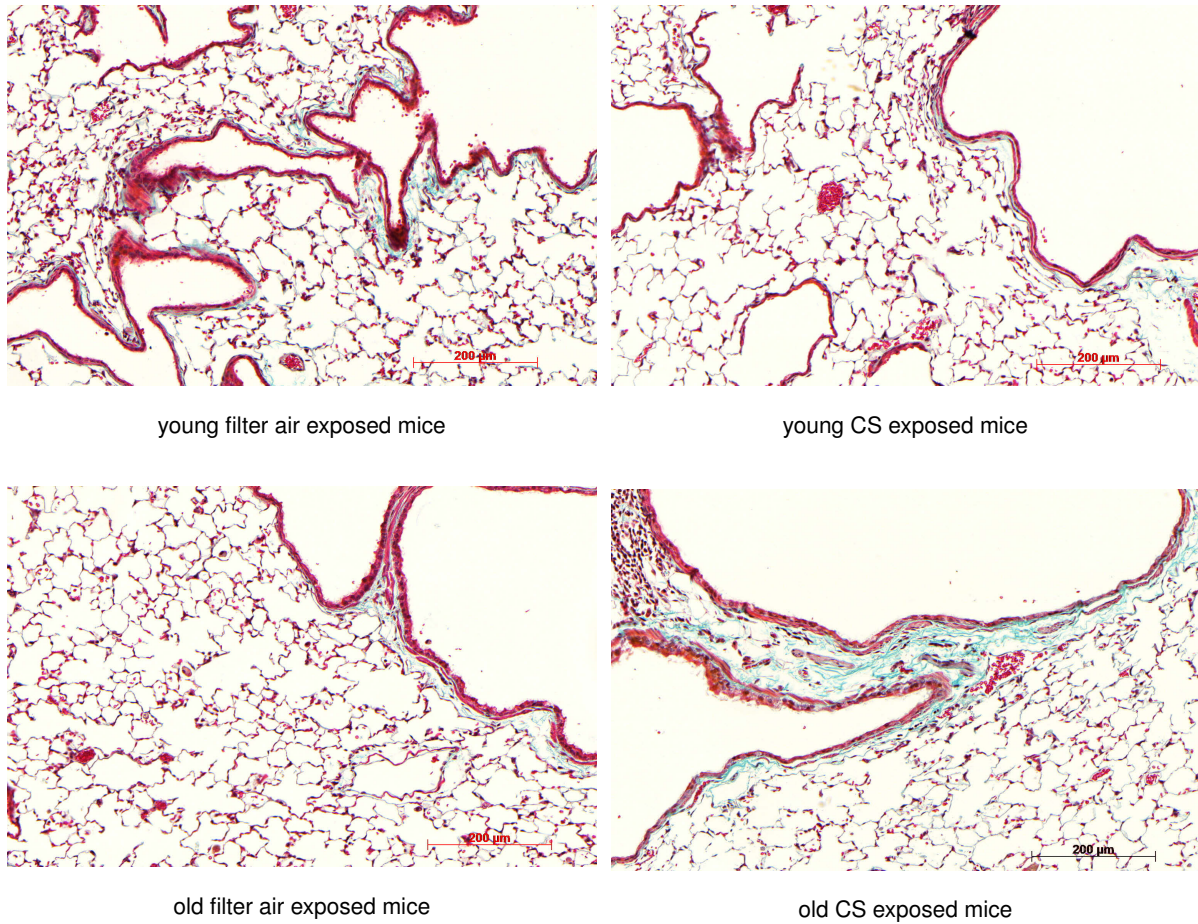
of these stainings were analysed as described in chapter 2.8.3 (figure 16). Here only a small, non-significant increase in airway collagen could be detected in the young animals after three months of cigarette smoke exposure. In contrast to those findings, in the old animals there is a significant increase in airway collagen due to cigarette smoke exposure. In addition an increased baseline in the old animals can be detected, but it does not reach significant levels.



**Figure 15:** Mean chord length, determined by using the CAST-sytem as described in chapter 2.8.1. A significant increase due to cigarette smoke exposure is seen only in the case of the old mice. Additionally, a baseline increase in old filter air exposed mice can be detected.



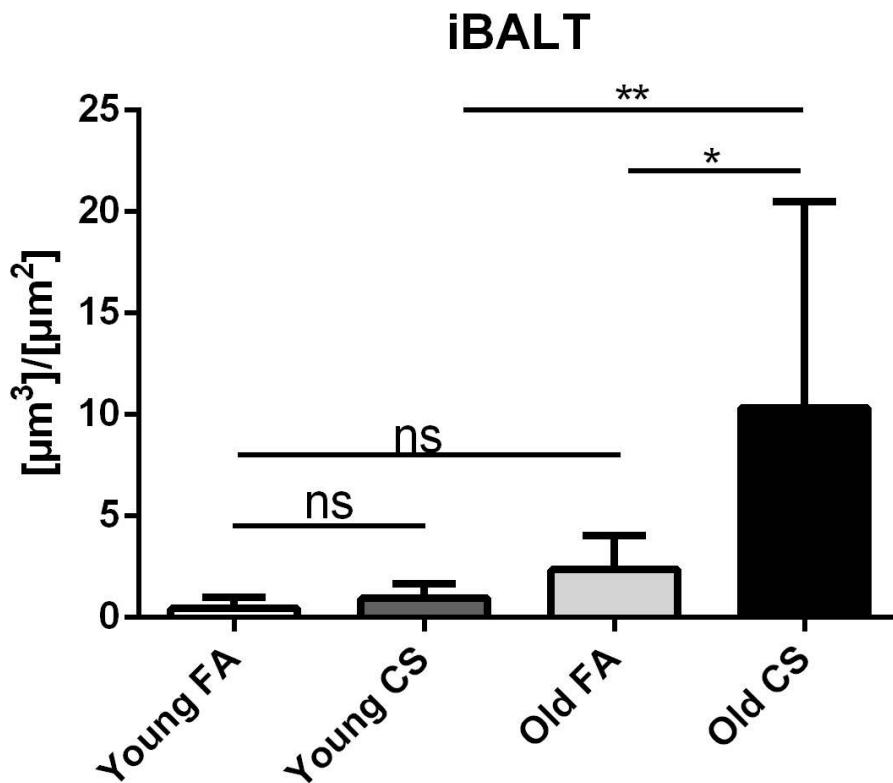
**Figure 16:** CAST-analysis of airway remodelling shows a significant increase due to cigarette smoke exposure in the old mice. In addition a significant difference can be seen in old and young cigarette smoke exposed animals.



**Figure 17:** Lung tissue stained with Masson's Trichrom staining as described in chapter 2.7.3 to show airway remodelling in the amount of collagen around the airways. A significant increase in collagen fibres (green) can only be detected in old cigarette smoke exposed animals.

### 3.2.3 Inducible Bronchus Associated Tissue

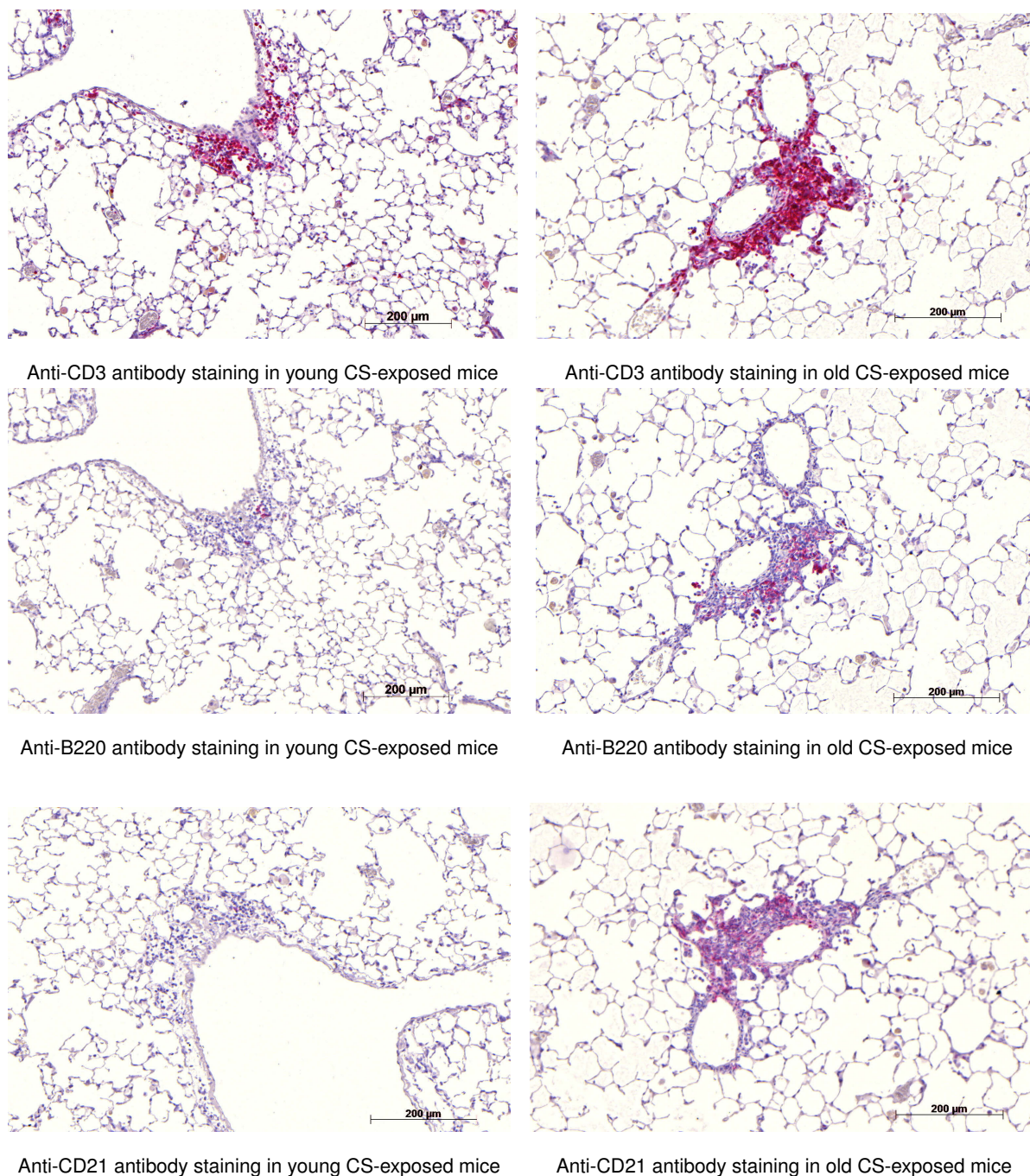
As described in previous papers [81] inducible Bronchus Associated Lymphoid Tissue (iBALT) plays a very important role in the development of COPD and the destruction of alveolar tissue in the progress of the disease. Figure 18 shows a significant increase in iBALT structures for cigarette smoke exposed old mice compared to old filter air exposed animals and to young cigarette smoke exposed mice. A slight, but not significant increase was visible in case of the young animals due to cigarette smoke exposure. Such slight changes can also be seen in the old filter air exposed animals where a negligible rise in the amount of iBALT structures was visible compared to both groups of young animals.



**Figure 18:** Volume of iBALT structures was determined by using the CAST-system as described in chapter 2.8.2. A significant increase in iBALT volume can be seen only in cigarette smoke exposed old animals compared to both, the filter air exposed peers and the cigarette smoke exposed young animals.

As we suspected these structures to be lymphoid tissue, immunohistochemical staining, as described in chapter 2.7.4, was performed to determine the dominating cell type in these structures. For staining, primary antibodies against CD3 positive T-cells, B220 positive B-cells as well as CD21 positive dendritic cells were used. In figure 19 it becomes obvious, that these structures are highly positive for all stainings in old cigarette smoke exposed mice, which strongly indicates that the structures in question indeed consist of lymphoid tissue. In young mice, these structures are much rarer and not as intensely stained as in the old animals.





**Figure 19:** Immunohistochemical staining of iBALT structures with different antibodies, as described in chapter 2.7.4. As iBALT structures could rarely be found in filter air exposed animals, the stainings are only presented from the lungs of cigarette smoke exposed animals. It can be seen that iBALT structures are stained positive for T-cells (CD3), B-cells (B220) as well as dendritic cells (CD21). These stainings are seen to be more intense in the older animals compared to the younger group.

### 3.3 Differentiation of T-cells

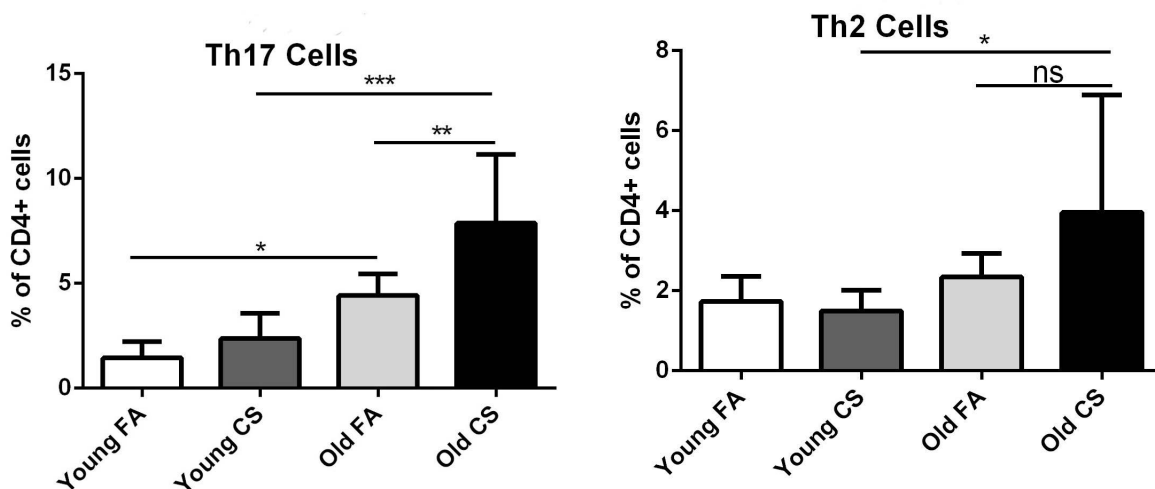
The analysis of BAL fluid shows an increase in different cell lines. The most prominent however was the increase in T-cells (see chapter 2.4). Thus a more differentiated analysis of subtypes seemed necessary. To accomplish this aim, we analysed CD4 positive T-helper cells taken from whole lung tissue that was prepared and analysed as described in chapter 2.6.

These analyses show the greatest increase in the subgroup of Th17 T-helper cells (figure 20). A strong increase of Th17 cells could be seen in the old CS-exposed mice compared to the filter air exposed animals. No difference due to cigarette smoke exposure could be found between the groups of young animals. Additionally a significant increase in the baseline levels of old filter air exposed animals compared to the younger peers could be determined (see figure 20).

The analysis of Th2 cells shows similar findings. A significant increase was detected in old compared to young CS-exposed animals. The slight increase in Th2 cells in old cigarette smoke exposed animals compared to the filter air exposed peer group does not reach statistical significance. Significant differences in base line levels were not found (see figure 21).

The analysis of Th1 cells showed no changes due to cigarette smoke exposure in both age groups. However the analysis of baseline levels between the two age groups showed an increase in the old FA exposed mice compared to younger peers (see figure 22).

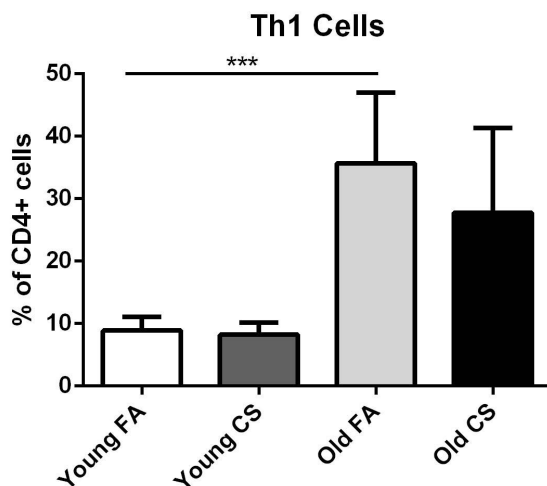
The last subtype that was analysed were regulatory T-cells. In this analysis an increase in numbers could only be shown for old CS-exposed mice. These differences however do not reach statistical significance. No changes could be found for the young CS-exposed mice compared to the filter air exposed group. Furthermore differences in baseline levels could not be detected (see figure 23).



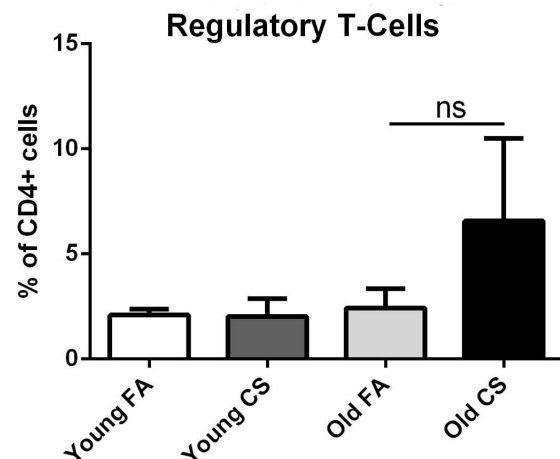
**Figure 20:** FACS Differentiation of Th17 CD4 positive T-helper cells: An increase can be seen in the old cigarette smoke exposed animals compared to both, the filter air exposed peer group and the cigarette smoke exposed young animals. Also a baseline increase in the old filter air exposed animals is found.

**Figure 21:** FACS Differentiation of Th2 CD4 positive T-helper cells: The analysis of Th2 cells shows a significant increase in the old cigarette smoke exposed mice in comparison to the young cigarette smoke exposed animals. Even though there is an increase also compared to the filter air exposed old mice, it does not reach statistical significance. No changes in baseline levels could be detected.





**Figure 22:** FACS Differentiation of Th1 CD4 positive T-helper cells: The only changes in the levels of Th1 are seen in the baseline levels. A significant increase could be found in the old filter air exposed animals compared to the younger peer group. No changes in Th1 levels due to smoking are seen.



**Figure 23:** FACS Differentiation of regulatory CD4 positive T-helper cells: The analysis of regulatory T-cells only shows an increase of regulatory T-cells for the old cigarette smoke exposed animals. Due to small numbers this increase does not reach statistical significance. There are no changes in baseline levels.

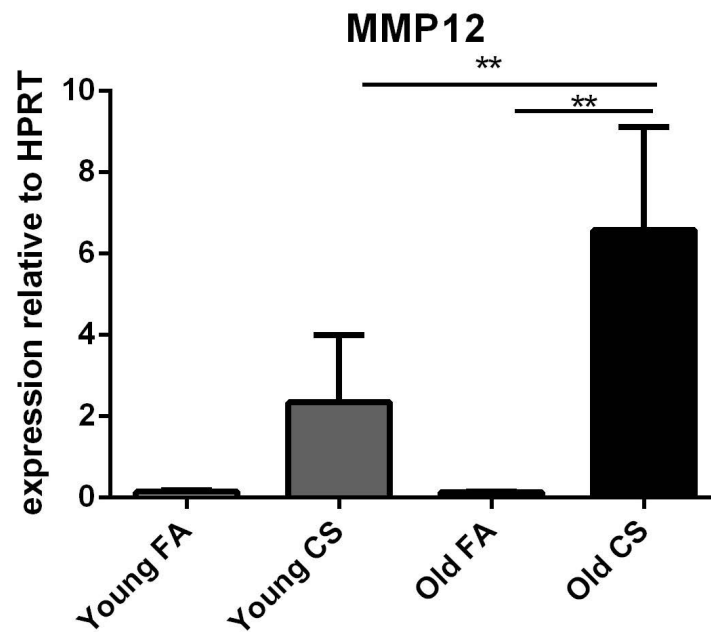
### 3.4 Macrophage Markers

As already described in chapter 1.2.1, activated macrophages play an important role in the development of COPD and emphysema.

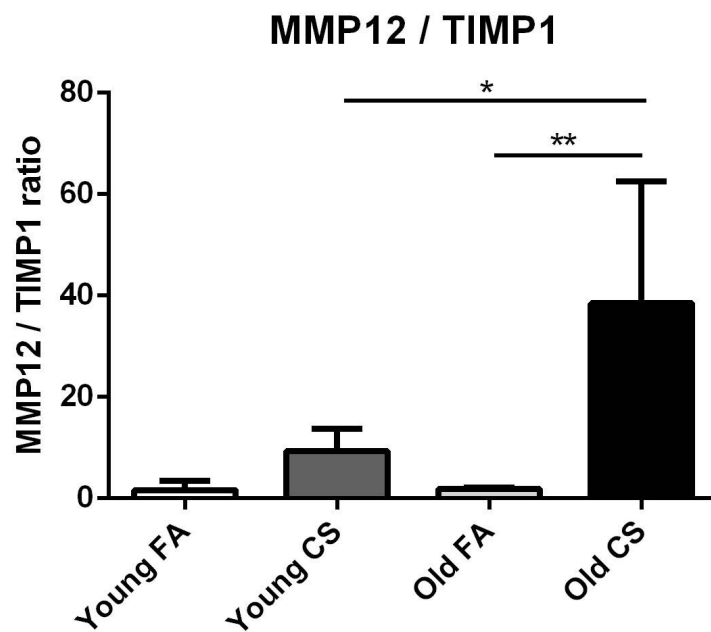
#### 3.4.1 MMP12-Matrix-Metallo-Proteinase 12

The most important marker for the activation status of macrophages is MMP12. To determine the amount of activated macrophages, we performed an immunohistochemical staining, as described in chapter 2.7.4 with the antibody given in table 13. We showed an increase in the amount of MMP12 positive macrophages in the young as well as in the old cigarette smoke exposed groups. This increase was greater in the older animals (see figure 26). To confirm these findings we also performed a qPCR analysis to determine the amount of MMP12 RNA as described in chapter 2.9 with the primers given in table 14. Figure 24 shows an increase in MMP12 expression in both old and young animals after CS-exposure. This increase has been shown to be significant in the old mice. The changes in case of the young animals do not reach statistical significance. No baseline differences between the filter air exposed groups was found (figure 24).

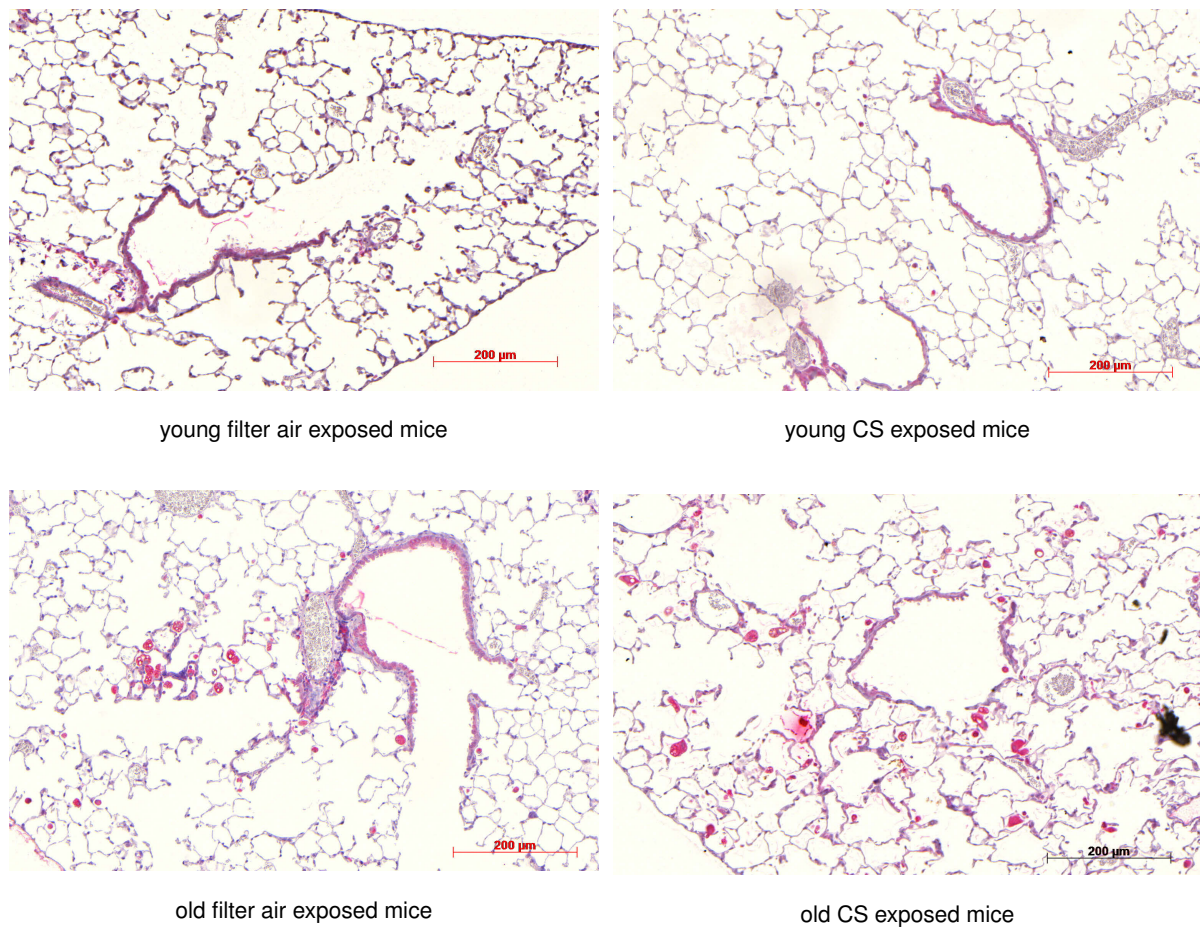
TIMP1 shows antagonistic effects to MMP12, so the effectiveness of MMP12 is also determined by the amount of TIMP1. Thus, a quantification of TIMP1 was performed as well (for primers see table 14), and the MMP12/TIMP1 ratio was determined. As no changes in TIMP1 could be found in any of the groups, the MMP12/TIMP1 ratio mirrors the results we have already seen in the analysis of MMP12 alone (figure 25).



**Figure 24:** qPCR of MMP12 as described in chapter 2.9: An increase of MMP12 RNA was found in the old cigarette smoke exposed animals compared to both, the filter air exposed peer group and the cigarette smoke exposed young animals. The slight increase in younger mice due to cigarette smoke exposure does not reach statistical significance.



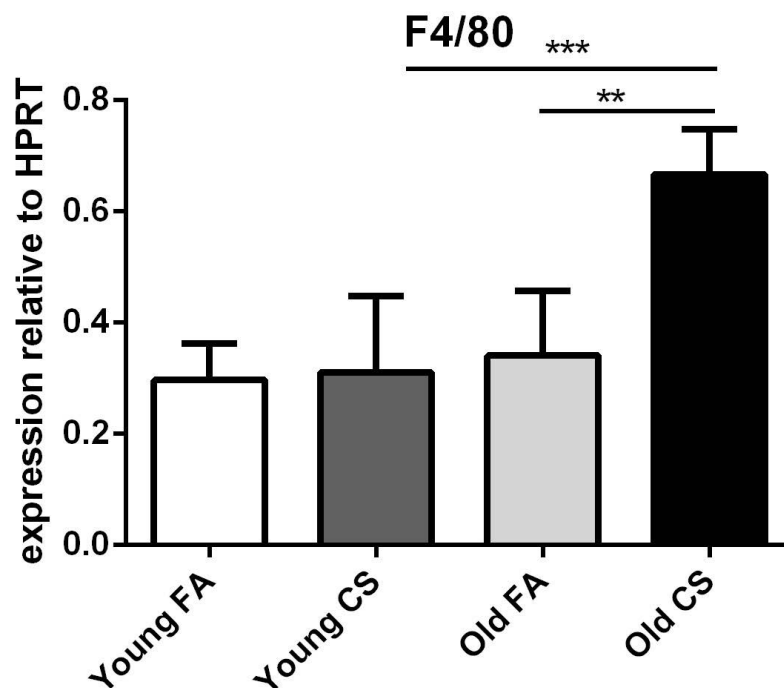
**Figure 25:** MMP12/TIMP1 ratio in lung tissue calculated from the results of qPCR as described in chapter 2.9: An increase in the MMP12/TIMP1 ratio was found only in the old cigarette smoke exposed mice compared to both, the young cigarette smoke exposed animals and the filter air exposed peer group.



**Figure 26:** Lung tissue of the mice after immunohistochemical staining with MMP12 antibody (see table 13) as described in chapter 2.7.4: An increase in MMP12 positive (red) macrophages can be seen in the old cigarette smoke exposed animals compared to both the cigarette smoke exposed young animals as well as the filter air exposed peer group.

### 3.4.2 F4/80

Another important macrophage marker is F4/80, which is a surface protein of macrophages [111]. In order to determine changes in its expression due to cigarette smoke, and thus the number of macrophages, a qPCR on total lung homogenates was performed with the primers given in table 14. This analysis resulted in a significant increase in F4/80 expression only for the CS-exposed old animals, while there are no changes due to cigarette smoke exposure in the young animals. One thus finds a significant difference between the old and young smoke exposed animals. No differences in baseline values for the FA groups could be detected, thus suggesting an increase in lung macrophages only after CS-exposure in older animals (figure 27).



**Figure 27:** qPCR of F4/80 as described in chapter 2.9: An increase in the expression of F4/80 is seen in the old cigarette smoke exposed mice both in comparison to the young cigarette smoke exposed animals and the filter air exposed peer group. No changes due to cigarette smoke exposure could be detected in the young animals. There are no differences in the baseline levels between the filter air exposed groups.

### 3.5 Chemoattractants

#### 3.5.1 Monocyte chemoattractant protein 1

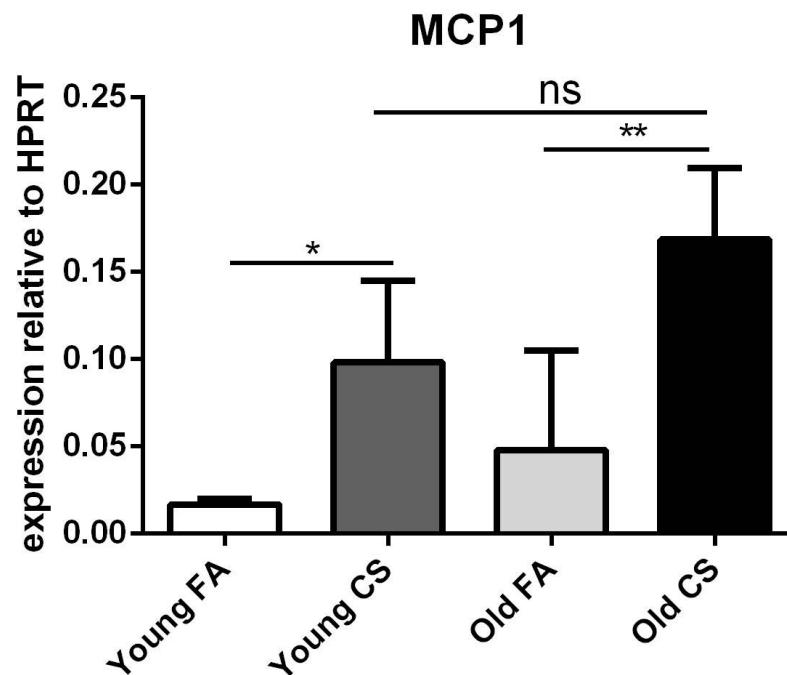
MCP-1 (CCL2) is a chemoattractant secreted predominantly by macrophages, although many cell types can secrete it, to recruit monocytes to sites of inflammation, where they can mature into macrophages [112]. Levels of MCP-1 mRNA in bronchiolar epithelial cells were shown to correlate with the number of macrophages in airway epithelia [112]. As discussed before, macrophages play an important role in the development of COPD (see chapters 1.2 and 3.4) and therefore recruitment markers were determined in our experiment as well.

In the qPCR performed on whole lung tissue (as described in chapter 2.9 with the primers in table 14) an increase in MCP-1 mRNA has been found in both smoking groups compared to the reference groups. The increase seems somewhat higher in the old CS-exposed animals, but there is no statistically significant difference between the two groups exposed to cigarette smoke. No baseline differences have been detected between old and young mice (figure 28).

#### 3.5.2 CXCL1 (C-X-C motif ligand 1)

CXCL1 has been shown to be an essential factor for neutrophil recruitment. An increase in neutrophils in the tissue of COPD patients has been demonstrated [59], even more so in COPD patients that in addition show chronic bronchitis.

In the analysis of CXCL1 in whole lung tissue using qPCR (as described in chapter 2.9) a significant increase of CXCL1 in the old CS-exposed animals, compared to filter air exposed



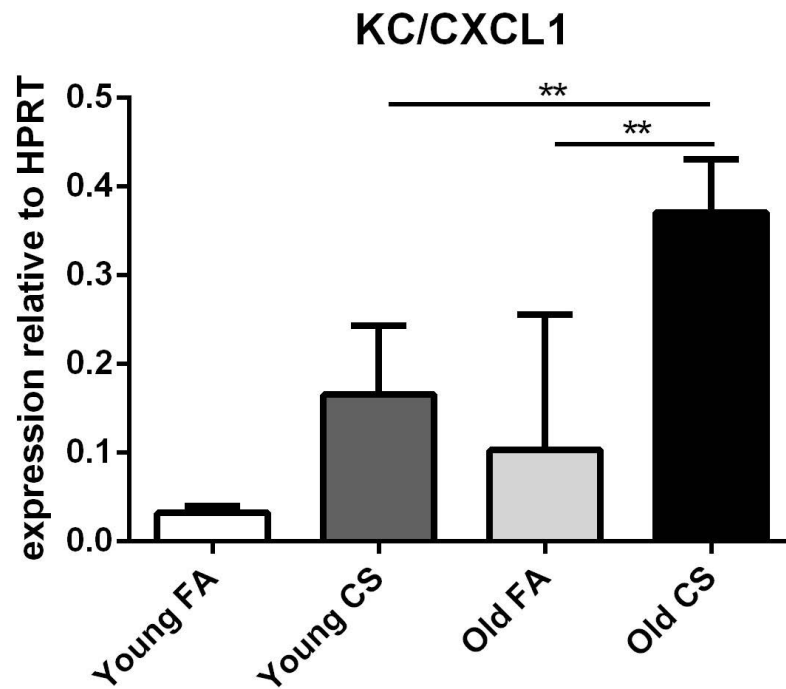
**Figure 28:** qPCR for MCP-1 in whole lung tissue as described in chapter 2.9: Increases in MCP-expression are seen in both cigarette smoke exposed age groups compared to the filter air exposed peers. The differences between the old and young cigarette smoke exposed groups are not significant. No differences in baseline levels are found.

control animals, has been detected. While the increase in the young CS-exposed animals is not statistically significant, the difference between the increase in the old and young CS-exposed mice is. Differences in base line levels could not be detected (figure 29).

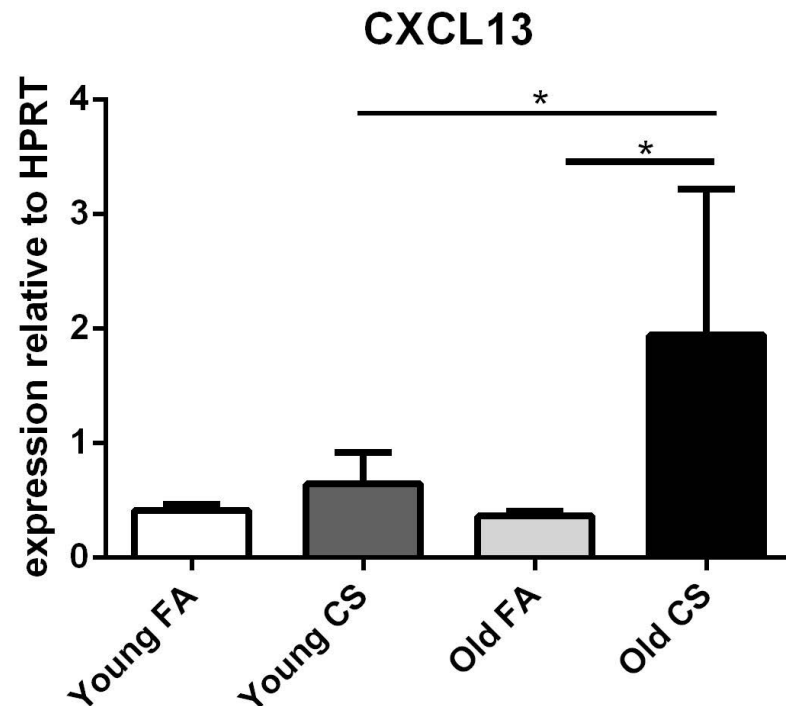
### 3.5.3 CXCL-13 (C-X-C motif ligand 13)

CXCL13 is the most important B-cell chemoattractant and plays a key role in the development of lymphoid follicles due to cigarette smoke exposure, as a lack of CXCL13 prevents lymphoid follicles from forming and shows a protective effect in the development of COPD [113].

In the qPCR analysis of CXCL13 in whole lung tissue (as described in chapter 2.9) the only significant change in expression levels was seen in the old CS-exposed mice compared to both the filter air exposed old mice and young CS-exposed animals (figure 30). There were no changes in baseline levels between old and young animals.



**Figure 29:** qPCR for CXCL1 in whole lung tissue as described in chapter 2.9: Increases in CXCL1 expression levels could be seen in the old cigarette smoke exposed animals compared to both, the filter air exposed peer group and the cigarette smoke exposed young mice. Differences in baseline levels could not be detected.



**Figure 30:** qPCR for CXCL13 in whole lung tissue as described in chapter 2.9: Increases in CXCL13 levels have been detected in the old cigarette smoke exposed animals compared to all peer groups. No differences in baseline levels could be detected.

### 3.6 Inflammation- and differentiation markers

To verify the results obtained in the analysis of iBALT and to further quantify inflammation, the expressions of inflammation markers as well as transcription factors for T-cell differentiation were determined.

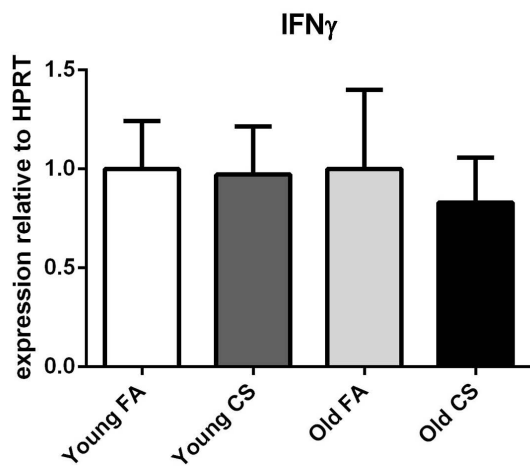
In the analysis of interferon  $\gamma$ , no changes in RNA expression due to cigarette smoke could be determined in either the old nor the young animals. Changes in the baseline levels between the different age groups could not be seen (see figure 31).

As a Further inflammation marker that was investigated was Interleukin 13. In this analysis a significant increase in the old CS-exposed mice could be detected. This increase was shown to be significant both in comparison to the filter air exposed control group as well as to the young CS-exposed mice. No changes could be determined in the young animals due to CS-exposure. Differences in baseline levels between the two filter air exposed groups could not be found (figure 32).

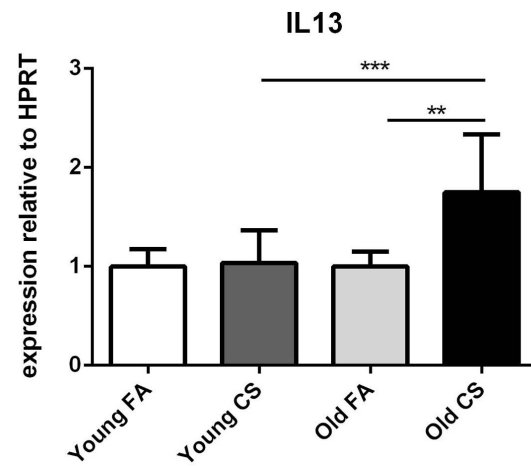
In addition, a determination of the expression of Gata3, a regulator for the differentiation of Th2 cells was performed. Even though the number of Th2 cells increased due to cigarette smoke, the expression of Gata3 slightly decreased in the cigarette smoke exposed animals compared to the respective filter air exposed groups. These trends however are not statistically significant. Differences in baseline levels of Gata3 in the filter air exposed groups could not be detected (figure 33).

The next transcription factor that analysed was Ror $\gamma$ t, that promotes differentiation of T cells into Th17 cells. Here we see results similar to the analysis of the amount of Th17 cells. The analysis showed a significant increase of Ror $\gamma$ t in the old CS-exposed group compared to the respective filter air group. This increase could also be shown in comparison to the young CS-exposed animals. No changes could be seen in the young mice due to cigarette smoke exposure. Differences in the baseline levels of both filter air exposed groups could not be detected (see figure 34).

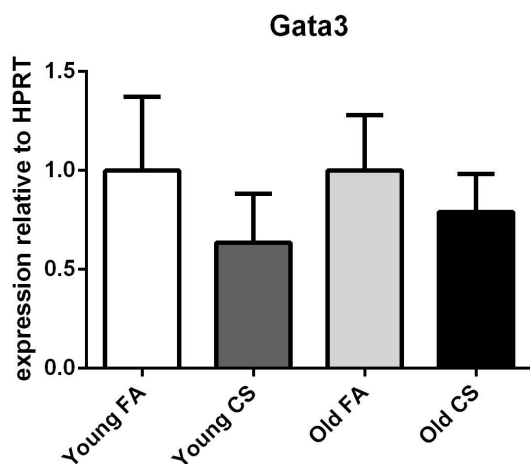
The last differentiation marker that was determined was Foxp3. Foxp3 is responsible for the differentiation of T-cells into regulatory T-cells. In this analysis a slight increase in expression in the old CS-exposed animals was visible, but the comparison with the filter air exposed animals of the same age group showed no significance. No trends could be seen in any of the other groups, neither between the young filter air exposed animals and the respective CS-exposed groups, nor in the baseline levels of the young and old filter air exposed groups (figure 35).



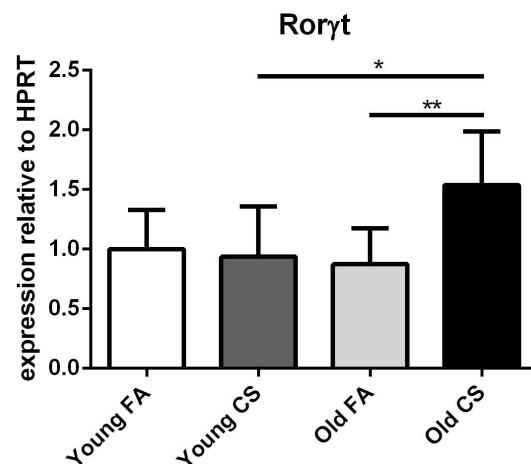
**Figure 31:** qPCR of Interferon- $\gamma$  expression: No changes due to cigarette smoke exposure or age could be detected.



**Figure 32:** qPCR of IL13 expression: An increase in IL13 expression is seen in the old cigarette smoke exposed group compared to all peer groups.

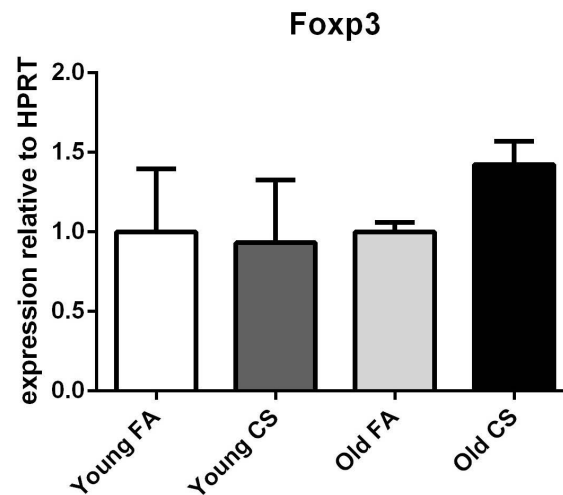


**Figure 33:** qPCR of Gata3 expression: Slight decreases in Gata3 levels due to cigarette smoke were found, but did not reach statistical significance. No changes in baseline levels are seen.



**Figure 34:** qPCR of Roryt expression: An increase in Roryt expression was found in the old cigarette smoke exposed animals compared all peer groups.





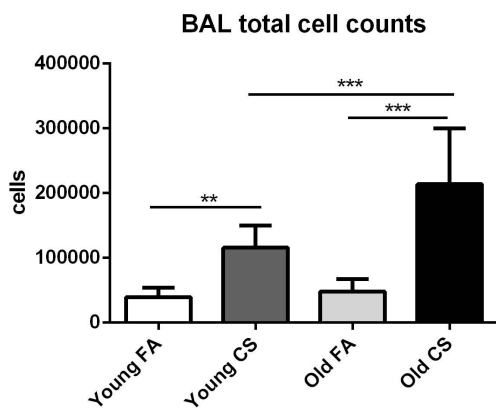
**Figure 35:** qPCR of Foxp3 expression: A slight increase in Foxp3 expression could be seen in the old cigarette smoke exposed group, but it does not reach statistical significance. No changes in baseline levels could be detected.

### 3.7 BAL-Cell-Differentiation

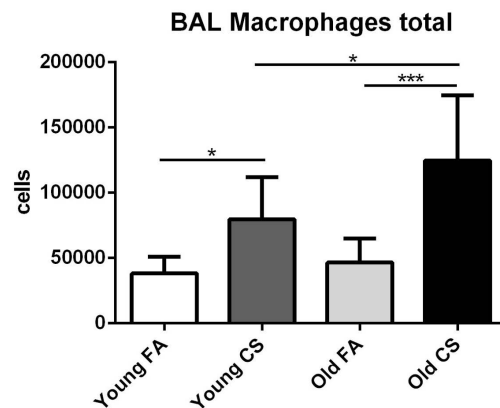
Bronchioalveolar lavage was performed on the animals (as described in chapter 2.4) and slides were prepared as discussed in chapter 2.7.1.

In the analysis of total cell counts, a significant increase in total cell count number between the groups of young mice as well as for the cigarette smoke exposed mice has been found, both for the young and old animals. Although both groups show a significant increase, there is still a greater increase in total cell counts in the group of old CS-exposed mice in comparison to young CS-exposed mice. No difference in base levels in the filter air exposed animals could be detected (see figure 36).

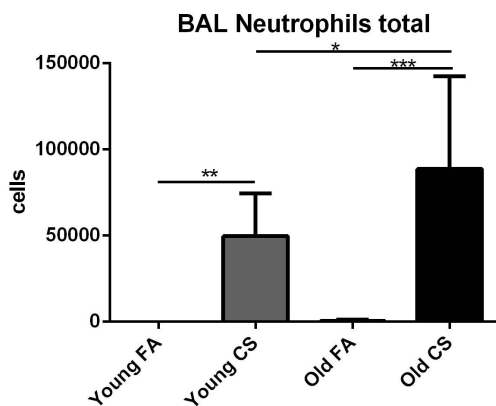
To further distinguish between the different cells types, differential cell counts were performed, as described in chapter 2.4. In differential cell counts, the same trends are seen in both macrophages (see figure 37), which are the dominant cell type, as well as in neutrophils (see figure 38). Also here no differences in base line levels could be detected. The cell line that has been shown to be the most important one in the development of COPD is lymphocytes (see chapter 1.2). In cell counts for lymphocytes, a significant increase has been found in the old CS-exposed animals compared to the control group, while a slight increase in the young cigarette smoke exposed mice compared to filter air exposed animals occurs with no statistical significance (figure 39). The difference in the increase between old and young CS-exposed animals is significant for the number of lymphocytes, while no baseline difference between the filter air exposed animals can be seen.



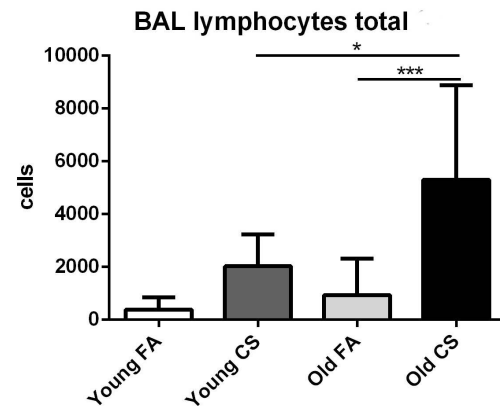
**Figure 36:** Total cell counts performed on BAL fluid: An increase in total cell counts is seen in both cigarette smoke exposed groups, with a larger increase in the old cigarette smoke exposed group. No differences in baseline levels are detected.



**Figure 37:** Total macrophage cell count in BAL fluid: An increase in total macrophage count could be found in both cigarette smoke exposed groups. The increase is larger for the old cigarette smoke exposed animals. No differences in baseline levels are found.



**Figure 38:** Total neutrophil cell count in BAL: The greatest increase could be seen in the old cigarette smoke exposed mice. An increase is also seen for the cigarette exposed young animals. The number for the filter air exposed groups is insignificant.



**Figure 39:** Total lymphocyte cell count in BAL: A significant increase in total lymphocyte count is seen only in the old cigarette smoke exposed animals. No differences in baseline occurred.

### 3.8 Ageing markers

#### 3.8.1 p16

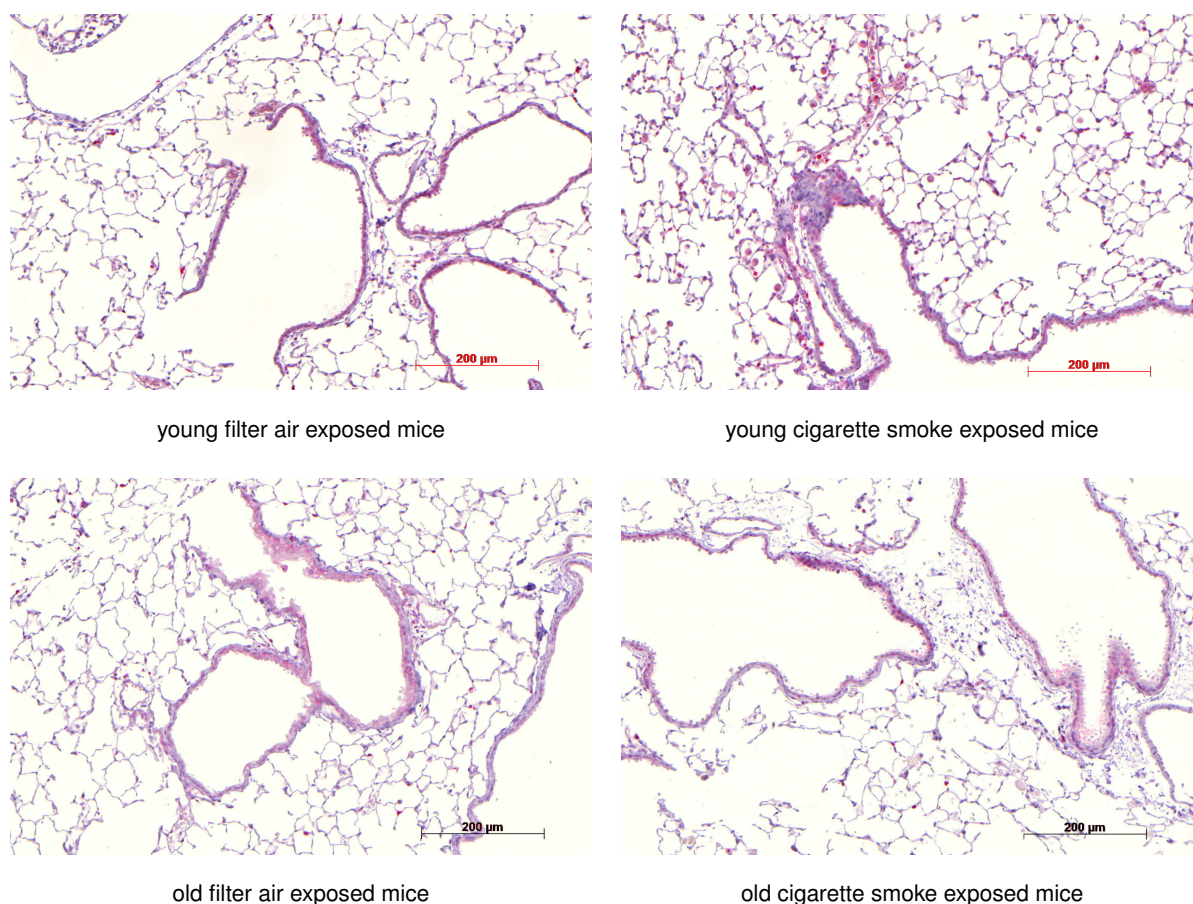
p16 has been described to play an important role in the development of tumours as well as to increase with cellular senescence [114],[115]. In this context it can also be considered as a marker of senescence. Therefore, an immunohistochemical staining for p 16 was performed (as described in chapter 2.7.4) with the antibody given in table 13. The analysis of the corresponding slides (see figure 40) suggested an increase in p16 due to cigarette smoke in both old and young animals. In light of these findings further analysis was performed.

To determine the changes in p16 expression more accurately, a qPCR analysis was made (as

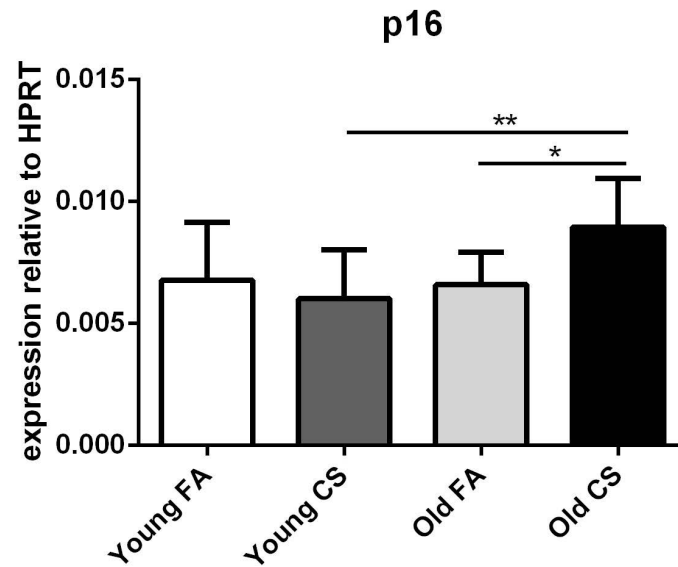
described in chapter 2.9 with the p16 primers given in table 14).

The analysis shows an increase in the old smoke exposed mice compared to the filter air exposed animals of the same age group. The analysis also exhibits a significant increase in the old CS-exposed mice compared to the young smoke exposed animals. The latter on the other hand do not show any changes compared to the respective filter air group. Also no changes in baseline levels of p16 could be detected (see figure 41).

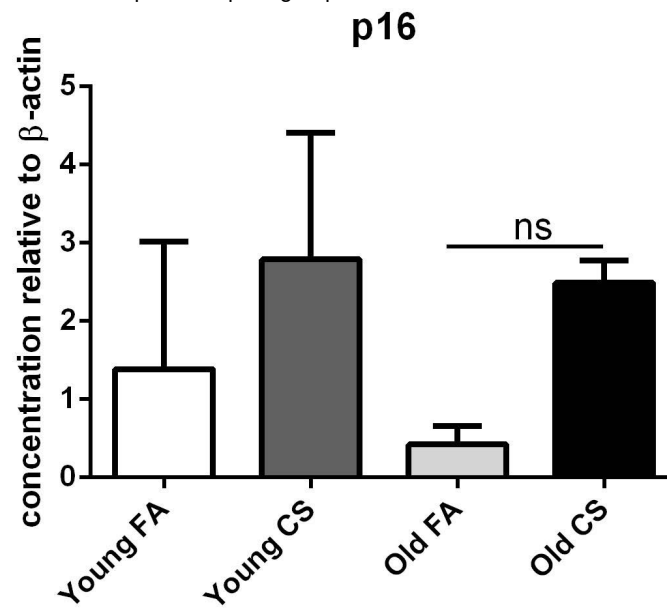
To further verify these results, additionally a western blot was done to not only investigate mRNA expression, but also the protein expression of p16. Western blot was performed as described in chapter 2.10, and a picture of the analysed membrane with markers and respective  $\beta$ -actin expression are shown in the attachment. Even though the changes are not found to be significant in the analysis of the western blot, there was a slight increase in p16 in both CS-exposed groups compared to filter air exposed animals.



**Figure 40:** Lung tissue of the mice after immunohistochemical staining with p16 antibody as described in chapter 2.7.4: An increase in p16 positive cells (red) in the old cigarette smoke exposed animals compared to the other groups can be seen.



**Figure 41:** qPCR analysis of p16 in whole lung tissue as described in chapter 2.9: A significant increase is seen in the old cigarette smoke exposed mice compared all peer groups. No differences in baseline levels could be detected.

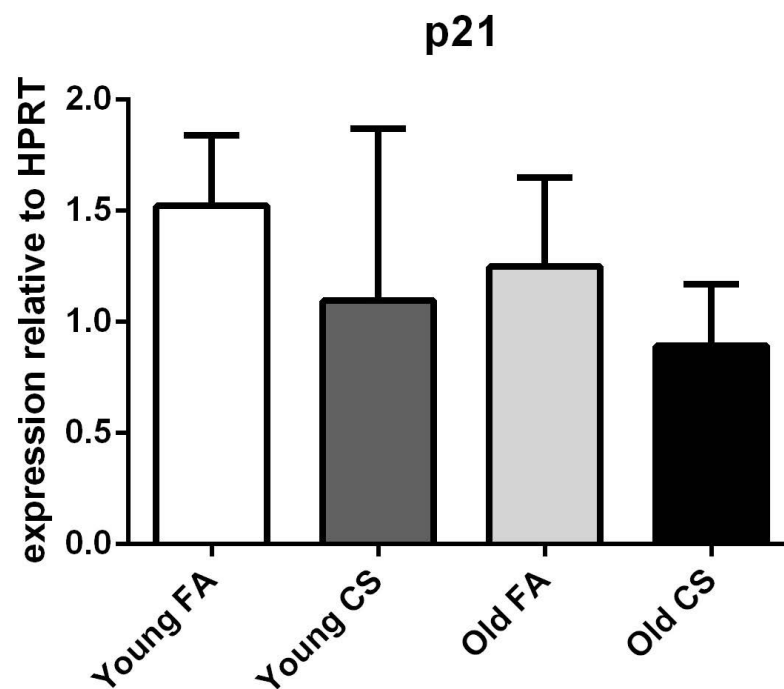


**Figure 42:** Analysis of Western Blot of p16 in comparison to  $\beta$ -actin (see figure ??): Due to the small numbers of animals analysed, the increases in the cigarette smoke exposed groups do not reach statistical significance

### 3.8.2 p21

Another marker for cellular senescence is p21. To quantify changes in p21 expression due to cigarette smoke, a qPCR analysis was performed as described in chapter 2.9 with the primers listed in table 14.

The results of this analysis show (see figure 43) a slight decrease of p21 due to cigarette smoke in both, the old and the young animals. These changes however are not statistically relevant. Differences in baseline levels between the filter air exposed young and old animals could not be detected.



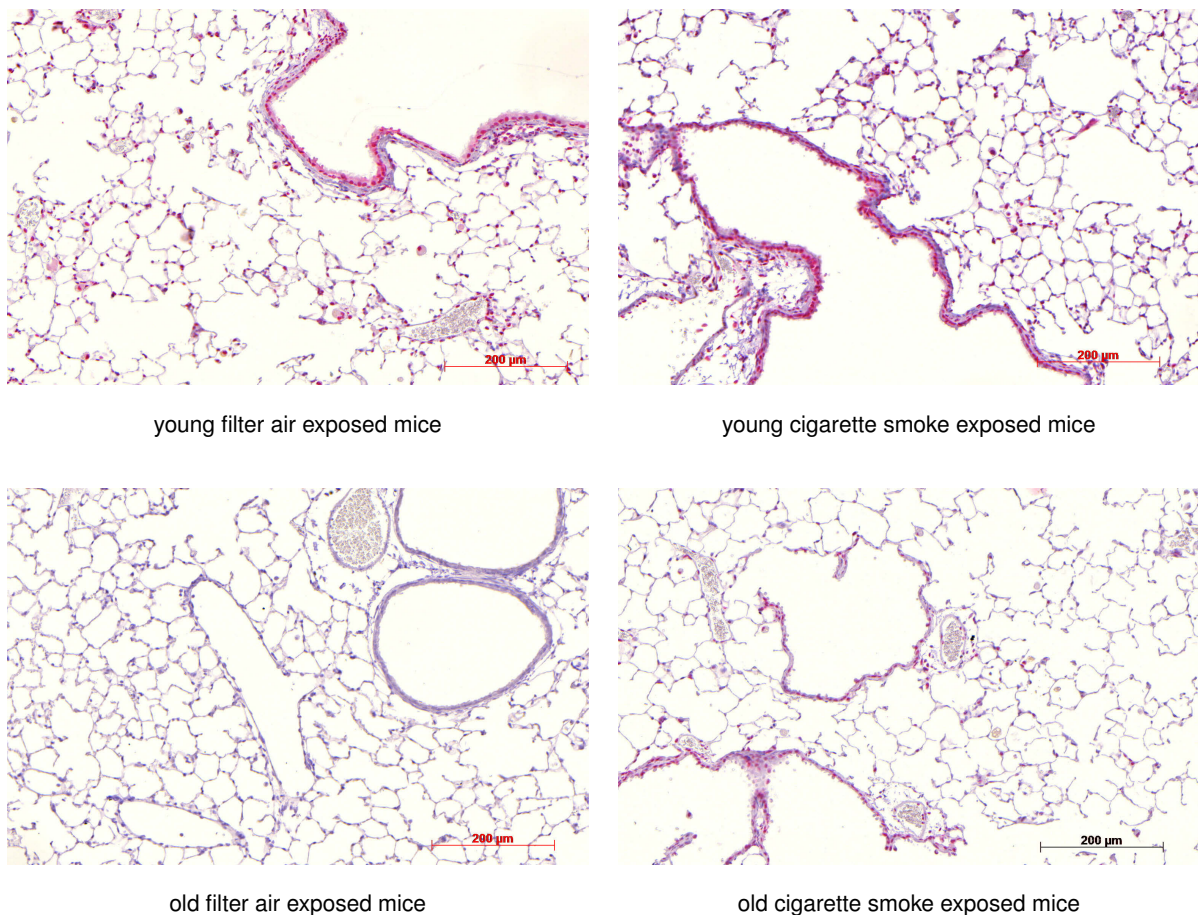
**Figure 43:** qPCR analysis of p21 in whole lung tissue as described in chapter 2.9: A slight decrease in p21 levels are visible for both cigarette smoke exposed groups, but did not reach statistical significance. The same goes for the decrease in baseline levels for the old filter air exposed animals.

### 3.8.3 SIRT1

SIRT1 is another important ageing marker. Thus an immunohistochemical staining of lung tissue with an anti- SIRT1 antibody was performed. As visible in the resulting pictures (see figure 44), the intensity of the red staining is decreased in the old animals, for both the filter air and cigarette smoke exposed groups, when compared to younger peers. Additionally a decrease due to cigarette smoke exposure is visible.

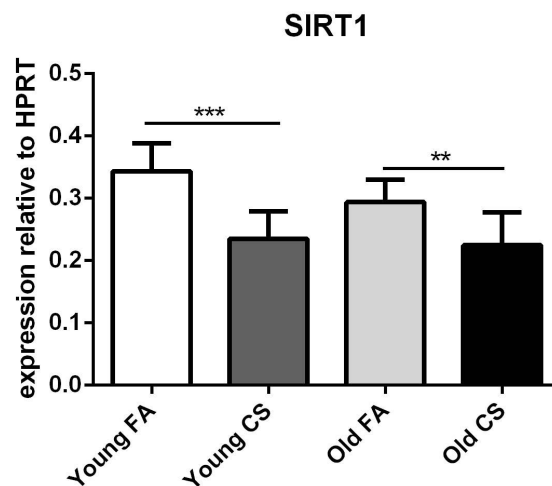
To further quantify the changes in SIRT1 expression, a qPCR of whole lung tissue was performed as described in chapter 2.9 with the primers given in table 14.

The results show a significant decrease in both, the young and the old smoke exposed animals compared to the respective filter air control groups. No differences in baseline levels was found. While there is a decreasing trend visible in the old filter air exposed animals compared to young animals, it does not reach statistical significance (see figure 45).

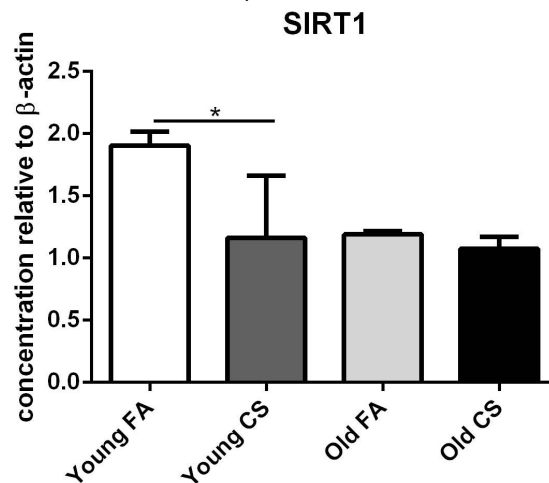


**Figure 44:** Lung tissue of the mice after immunohistochemical staining with SIRT1 antibody as described in chapter 2.7.4: A decrease in Sirt1 expression in the older animals can be seen. Additionally the SIRT1 expression seems to be decreased in the cigarette smoke exposed groups.

To validate the PCR results, a western blot was additionally performed. Also in these results, a significant decrease of SIRT1 in the young cigarette smoke exposed mice compared to the filter air exposed animals has been detected. No differences can be seen in the old cigarette smoke exposed group compared to old filter air exposed animals as the old filter air exposed animals



**Figure 45:** qPCR analysis of SIRT1 in whole lung tissue as described in chapter 2.9: A significant decrease of SIRT1 due to cigarette smoke exposure can be seen in both, the old and the young animals. Additionally there seems to be a slight decrease in baseline levels in the older filter air exposed animals, that does not reach statistical significance.



**Figure 46:** Analysis of Western Blot of SIRT1 in comparison to β-actin: A decrease in SIRT1 is seen in the young animals due to cigarette smoke exposure. The decrease in baseline levels in old filter air exposed animals does not reach statistical significance. This might be attributed to the low animal numbers that were tested.

have already reached the lowest expression levels of SIRT1. In accordance with those findings, there was a decrease in baseline values of SIRT 1 in the old filter air exposed animals compared to the young mice (figure 46).





## 4 Discussion

### 4.1 The use of a mouse model to simulate COPD development

Epidemiologic studies have shown, that COPD is a disease that mainly occurs in old patients (e.g. [7]). The aim of this thesis was to investigate if ageing itself plays an important role in the development of COPD. In that case, the age distribution of COPD patients would not only be a cumulative effect of cigarette smoke exposure. To discriminate the effect of smoke from the influence of ageing, we exposed old and young mice to mainstream cigarette smoke. This form of cigarette smoke exposure is the one that comes closest to mimic human smoking habits. After three months of cigarette smoke exposure we noticed a decline in lung function, defined by an increase in compliance, in the old animals, while there were no changes in the young animals. In a stereological analysis of lung tissue we found however the development of emphysema in both groups of aged mice, i.e. also for those only exposed to filter air only. This finding is similar to senile emphysema described for humans: an age dependent decrease in lung function as well as an enlargement in airspace have been observed for healthy elderly humans compared to young controls. In contrast to emphysema development in COPD patients, senile emphysema is characterized by airspace enlargement without destruction of the alveolar walls [116]. However even though aged mice already show signs of emphysema development without being exposed to cigarette smoke, the increase in airspace enlargement due to cigarette smoke exposure is still significant. In addition to the development of emphysema, we have further shown that in old animals the amount of collagen surrounding the airways increases due to cigarette smoke exposure. The latter is similar to the findings in human COPD patients where the total wall thickening as well as the thickening of each of the airway wall compartment show a strong association with COPD progression as defined by the GOLD stages [1].

We should point out, that in contrast to our results Zhou et al. have reported [117] that after 6 months of cigarette smoke exposure old mice did not show enhanced emphysema compared to young animals. These deviating results might be due to significant differences. Although the same mouse strain and the same cigarettes have been used, the method of cigarette smoke exposure was different. While in our experiments a smoke chamber for whole body exposure with 20 cigarettes per day has been chosen, in [117] a nose only exposure with three cigarettes per day was used. The latter might also account for the different cigarette smoke exposure times required to develop emphysema. The long duration of the experiment required in [117] is probably responsible for the missing age effect in this study. This hypothesis is supported by a set of further experiments performed in our group. Here young mice exposed to cigarette smoke for four months showed similar airspace enlargement as old mice did after three months of cigarette smoke exposure [81]. Furthermore, unpublished data from our group show no difference in airspace enlargement for old and young mice that were exposed to cigarette smoke for four months. Thus, we conclude that the results in [117] are not in contradiction with our findings of an age effect after three months of cigarette smoke exposure.

## 4.2 The role of inflammation in the development of COPD

In the analysis of bronchoalveolar lavage fluid we have found an increase of total cell number in both smoke exposed groups of mice. This effect has been much larger however for the old animals. These findings are indicative of a more pronounced immune system activation in the older mice. Since changes in the immune system due to ageing are well known, we performed an analysis of the immune response of old animals compared to younger animals. We have found an increase in inducible bronchus associated lymphoid tissue iBALT structures in old animals exposed to cigarette smoke that could not be seen in any of the other groups. Such iBALT structures are known to play an important role in the development of COPD. Hogg et al. [1] have shown an association between the number of small airways containing lymphoid follicles and the severity of the disease, defined by lung function analysis according to GOLD. These findings could at least in part be attributed to an increase in collagen fibres in the subepithelium of bronchioles occurring around these lymphoid structures [118].

Immunohistochemic staining of the iBALT structures in our experiment unveiled, that they mainly consist of B-cells and T-cells as well as to a smaller amount, of dendritic cells. Both B- and T-cells are well known to play a major role in the development of COPD [81].

Increased amounts of B-cells have been found in small airways of COPD patients [1] as well as in biopsies of large airways of patients with COPD. It has further been shown, that increases in the amount of B-cells in airways of COPD patients correlate with disease stage and lung function (FEV<sub>1</sub>) [79]. In addition to the increase of B-cells in the airways of COPD patients, infiltrates of B-cells were also found in lung interstitium. In a mouse model an increase in both, size and number of such infiltrates could be proven to be caused by prolonged smoking [80]. The importance of B-cells for both, the development of iBALT structures as well as for emphysema could be demonstrated by cigarette smoke exposure of B-cell deficient mice [81]. In these animals, an inability to form iBALT structures as well as an immunity to cigarette smoke induced emphysema could be observed. This immunity to emphysema seems to be caused by an inability of these animals to activate macrophages. In vitro experiments have shown that B-cell derived IL10 is crucial for the activation of MMP12 expression in macrophages [81].

B-cells contained in iBALT structures produce CXCL13 which is a chemoattractant for other B-cells. In the whole lung tissue derived from our experiments we have seen an increased expression of CXCL13 for old cigarette smoke exposed mice only. This finding is consistent with the increased numbers of iBALT structures observed in these mice. Increased amounts of CXCL13 had already been described earlier, both in patients with COPD as well as in a CS-mouse model for COPD [113], [119]. It was shown that CXCL13 is required for B-cell migration into lung tissue and for the formation of iBALT. Since the B-cells contained in iBALT structures also produce CXCL13, a positive feedback mechanism gets established. Treatment with a CXCL13 antibody has prevented iBALT formation in prophylactic as well as therapeutic approach [119].

iBALT structures also contain T-cells. There are different subtypes of T-cells contributing to the development of COPD. It has been shown that cigarette smoke exposure can lead to defective T-cells, that may give rise to emphysema development as well as to formation of lymphoid follicles in immune deficient, but otherwise healthy animals, independent of cigarette smoke exposure. This effect however has so far only been found in T-cells isolated from lung tissue [71]. The effect of T-cells for emphysema development can also be caused by a changed chemokine profile as it has been found that T-cells isolated from COPD patients have a changed chemokine expression [120].

In the analysis of cells in bronchoalveolar lavage fluid we found an increase of total cell counts in

both of the cigarette smoke exposed groups compared to the respective filter air exposed groups. This increase was larger for the old smoke exposed mice than for the young ones. Further differentiation of BAL cells unveiled an increase of lymphocytes in the old smoke exposed animals only.

In our analysis of whole lung tissue, we found a significant increase in IL13 mRNA levels in old cigarette smoke exposed animals when compared to both, the respective filter air exposed group as well as the young cigarette smoke exposed group. The levels of IL13 in plasma of COPD patients has been shown to correlate to lung function impairment [120].

To further investigate the T-cell response occurring in lungs due to long term cigarette smoke exposure, the different subtypes of CD4 positive T-helper-cells in whole lung tissue were differentiated. An increase in Th1 cells could be detected in old mice exposed to cigarette smoke as well as in filter air exposed control animals. This leads to the assumption that the increase in Th1 cells is age dependent. Th2 cells showed an increase in old cigarette smoke exposed animals in comparison to young animals only, but no significant difference was found compared to the filter air exposed control group. This finding is consistent with in vitro studies that showed an increase in Th1 differentiation of human CD4+ cells after stimulation in comparison to cells taken from younger subjects [121]. In our experiment the most prominent changes regarding T-cells were found for the Th17 cells, that were significantly increased in the old smoke exposed animals compared to all other control groups. These results are consistent with previous findings, describing an increase in Th17 cells due to cigarette smoke [122]. Furthermore, the number of IL17 producing T-helper cells has also been described to increase with age. The up-regulation of Th17 cells is dependent on the retinoic acid related receptor  $\gamma$ t (ROR $\gamma$ t). Pharmaceutical suppression of ROR $\gamma$ t showed a decreased differentiation of CD4+ T-helper cells towards IL17 producing cells [122].

As we have already shown for the number of Th17 cells, ROR $\gamma$ t mRNA expression in our experiment has been found to be only increased in the old cigarette smoke exposed animals. These findings are consistent with the results of Chu et al. [123] who have demonstrated a significant increase of ROR $\gamma$ t in COPD patients. In addition, it has been shown that the ratio of Foxp3/ROR $\gamma$ t is related to both, emphysema development as well as lung function analysis. Corroborating these findings, the number of regulatory T-cells have been shown to decrease with age. Regulatory T-cells are responsible for the down regulation of Th17 cells and therefore have an anti-inflammatory effect [124]. They are thus important for regulating immune response and inflammatory homeostasis [125]. In our experiment there was a slight trend towards an up-regulation of regulatory T-cells for the old cigarette smoke exposed mice which is consistent with the findings of Schmitt et al. [124], who have demonstrated an increased up-regulation of regulatory T-cells upon stimulation. We have seen the same trend as for regulatory T-cells in the mRNA expression of Foxp3 in whole lung tissue where only a slight increase in old smoke exposed animals is visible, but does not reach statistical significance. Foxp3 is a specific transcription factor for regulatory T-cells and therefore is considered a reliable marker. [126] The much greater increase in Th17 cells compared to regulatory T-cells would lead to an increased expression of IL17. The role of IL17 has not yet been fully understood. It has been shown however, that iBALT development is dependent on IL17, as IL17-deficient mice develop only smaller and fewer iBALT structures after cigarette smoke exposure in comparison to wild type animals. IL17 has been shown to be only required for the development, but not for the maintenance of iBALT structures [127]. Since the development of iBALT structures also occurs in COPD it can be assumed that the TH17 cells play an important role in COPD development. In addition, it has been discovered that IL17 leads to an increased expression of matrix-metalloproteinases (MMP), in particular MMP12 as well as to a reduction of TIMP, an antagonist of

MMPs. As MMP12 is secreted by macrophages, focal accumulation of mononuclear cells as well as increased formation of lymphoid structures in mice over-expressing IL17 is to be expected and has been shown. Since MMP12 has been unveiled to be one of the main contributors to the development of emphysema, as expected mice unable to express IL17 show less emphysema development than wild type animals after chronic exposure to cigarette smoke. In these animals, also the total number of inflammatory cells in the lung after cigarette smoke exposure is decreased in comparison to wild type animals [128], [129].

In addition to the increase in lymphocytes in bronchioalveolar lavage, we also found an increase in macrophages. Even though the number of macrophages is increased in both cigarette exposed groups, a significantly higher number has been detected in the old animals. Concomitant with these results, an analysis of macrophage chemoattractant protein 1 (MCP1) in BAL has shown an increase in both cigarette smoke exposed groups. MCP1 has been shown to be important for COPD development. It has been found to be increased in sputum of COPD patients and to correlate with FEV<sub>1</sub> [130]. In addition, a correlation between MCP1 expression from bronchial epithelial cells and the number of intraepithelial macrophages has been detected [131]. Alveolar macrophages have been shown to be vital for the development of emphysema. It has been demonstrated that mice deficient in matrixmetalloproteinases (MMP) are protected against cigarette smoke induced emphysema [65]. Since these mice also recruit significantly fewer macrophages to the alveoles, mice were instilled with MCP1 to stimulate the migration of macrophages into the lungs [65]. These findings show that it is not the macrophages themselves, but the MMPs they produce, that are responsible for the development of COPD. Therefore an analysis of MMP12 expression of macrophages was performed in our experiment. Immunohistochemic staining for MMP12 in lung tissue has shown more MMP12 positive macrophages in old cigarette smoke exposed mice than in any of the other groups. To confirm these findings, we performed a qPCR analysis of MMP12 mRNA expression in whole lung tissue. We have found an increase of MMP12 expression in old cigarette exposed mice compared to all other groups. Similar results could be seen in the analysis of the MMP12/TIMP1 ratio. Since TIMP1 is an inhibitor of all MMPs, an increase in TIMP1 expression would inhibit the development of emphysema [132]. Since no changes in TIMP1 expression could be detected, the increase in MMP12/TIMP1 ratio can be solely attributed to the increase in MMP12. To confirm these findings, another macrophage marker was analysed. F4/80 has been shown to be only expressed by macrophages, and is therefore a reliable marker [111]. Furthermore, F4/80 has also been found to be increased with maturation of macrophages [133]. In our experiment we have seen an increase in F4/80 in cigarette exposed old mice only, confirming an increase in mature alveolar macrophages. The last group of cells we investigated and that showed an increased number in the analysis of bronchoalveolar lavage, are the neutrophils. The total neutrophil count increased in both cigarette smoke exposed groups, but the increase was significantly higher in the old cigarette smoke exposed mice when compared to young animals. To further substantiate these findings, an analysis of mRNA expression of C-X-C motif ligand (CXCL1) was performed. As with the total number of neutrophils, an increase could only be seen in the old cigarette smoke exposed mice compared to the respective control groups as well as the young cigarette smoke exposed animals. CXCL1 has been shown to be a powerful chemoattractant for neutrophils. It has been demonstrated that chemotaxis of neutrophils is dependent on CXCL1 and CXCL2 [134]. Not only macrophages, but also neutrophils are important contributors to the development of emphysema [135]. It has been shown that mice deficient in neutrophil elastase have decreased airspace enlargement compared to wild type animals following cigarette smoke exposure. In addition, neutrophil elastase proteolytically activates MMP12 and degrades its inhibitor TIMP1 [135].

### 4.3 The effect of ageing on the development of COPD

Aging does not only occur in individuals, but can also be seen on the cellular level. An increase in senescent cells in COPD patients compared to healthy non smoking as well as smoking control subjects has been described. Cellular senescence has several markers of which the most important ones that increase with age are p16 and p21. These markers have also been shown to be negatively correlated to telomere length, which is a marker for replicative senescence. The levels of cellular senescence markers have also been described to correlate with lung function impairment [136]. The cell type with the greatest change in p16 expression in patients with emphysema is type II alveolar cells [137]. Thus, in order to determine the changes in expression of these senescence markers in our work, first an immunohistochemical staining for p16 was performed. A slight increase in staining intensity could be seen in the old smoke exposed mice. To further substantiate these findings we performed an analysis of mRNA expression of p16. Here we determined a significant increase in expression levels only in old cigarette smoke exposed mice compared to both the respective filter air group and the young cigarette smoke exposed animals. Analysis of protein levels using western blot revealed no significant changes, but this might be attributed to a difficult analysis of a small protein. For p21, no significant changes could be detected. Chronic inflammation of the lung caused by cigarette smoke can cause premature ageing of lung tissue. Premature senescence under these circumstances leads to a decreased regenerative capacity that might contribute to emphysema development. It has been demonstrated that tissue ageing due to chronic inflammation can be inhibited by treatment with anti-inflammatory drugs [138].

Not only chronic inflammation is responsible for the premature ageing of the lung, but also the cigarette smoke itself. It has been shown that cells treated with cigarette smoke extract show signs of cellular senescence in combination with an irreversible growth arrest. Interestingly, these changes have been found not to be caused by nicotine alone [139]. In addition to cellular senescence caused by chronic inflammation, also replicative senescence due to an increased destruction of cells might play a role. High turnover of cells results in shortening of telomeres, which has been shown to cause increased emphysema development. In mice with shortened telomeres, greater DNA damage due to cigarette smoke exposure has been found. In addition, in mice with shortened telomeres, p21 fails to be down-regulated after smoking cessation, which might account in part for the continuous decline in lung function of COPD patients after smoking cessation. Mutations in telomerase genes have been found to be a risk factor for both, the development of COPD as well as pulmonary fibrosis [140].

The other important ageing marker that plays a role in COPD development is SIRT1. SIRT1 has been shown to decrease in mice exposed to cigarette smoke [141]. Similar results have also been shown for human smokers and COPD patients [142]. We have shown a decrease in SIRT1 in both cigarette smoke exposed groups, but have seen no changes between the old and the young animals. A decrease in SIRT1 has been found to predispose for emphysema development upon cigarette smoke exposure. In addition, overexpression of SIRT1 has been demonstrated to have a protective effect against the development of emphysema, making it a prime target for pharmaceutical approaches [141]. SIRT1 has been found to decrease autophagy which explains why decreased levels of SIRT1 attribute to the development of COPD [143].



## 5 Conclusion and Outlook

In conclusion, we have found that aged mice develop cigarette smoke induced emphysema at an earlier time-point of cigarette smoke exposure than younger animals do. The development of emphysema was substantiated by lung function analysis as well as stereological analysis of embedded lung tissue where we found an increased mean alveolar intercept. Since changes in lung function are usually attributed to small airway disease, we analysed the remodelling of small airways. Here we found an increase in airway collagen. One of the main theories for COPD development is that COPD might be caused by an immune response to cigarette smoke. We have shown an increased amount of bronchus associated lymphoid tissue (iBALT) as well as an increase in inflammatory cells in the bronchoalveolar lavage. The greatest changes were to be seen in the numbers of Th17 CD4 positive T-helper cells, the number and activation status (defined by the expression of MMP12) of macrophages and the number of neutrophils. All of these cell lines were increased in the lungs of old cigarette smoke exposed mice, but neither, or at least not as much, in the lungs of old filter air exposed controls nor in young cigarette smoke exposed animals. Changes in the immune response due to ageing have been described in humans as well as in mice. Thus, to determine whether the changes in the immune response we have seen in the old animals are due to cellular senescence, an analysis of ageing markers on lung tissue was performed. We have seen an increase in the ageing marker p16 in old smoke exposed mice, indicating an increased cellular senescence. In addition, a decrease in SIRT1 in both cigarette smoke exposed groups could be found.

For further investigations, the smoking experiment could be repeated using young mice that received a bone marrow transplant with aged cells. This could substantiate our hypothesis that an aged immune system predisposes for the development of cigarette smoke induced COPD. Another possibility would be to expose even older mice to cigarette smoke, as 12 month old mice would correlate to middle aged humans, that still rarely develop emphysema.





## 6 References

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## Eidesstattliche Versicherung

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Name, Vorname

Ich erkläre hiermit an Eides statt,

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